Internalisation of -MSH analogues to B16 murine melanoma cells via the -MSH receptor

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Award date:
1993

Awarding institution:
University of Bath

INTERNALISATION OF α-MSH ANALOGUES TO B16 MURINE MELANOMA CELLS VIA THE α-MSH RECEPTOR

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for the degree of

Doctor of Philosophy

of the University of Bath

1993

This research was carried out in the School of Pharmacy and Pharmacology of the University of Bath

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ABSTRACT

The major aim of the work presented in this thesis relates to site-specific drug delivery of peptide analogues. A model system was used to study the binding and subsequent uptake of α-MSH analogues into B16 murine melanoma cells by receptor-mediated endocytosis. An acid wash technique was introduced to differentiate between extracellularly bound and internalised ligand. An additional area of work was the fate of the ligands once they were inside the cells.

The superpotent α-MSH analogue, Ac-[Nle^4,D-Phe^7]α-MSH, was labelled with ^{125}I and bound to B16 murine melanoma cells. At 4°C, no internalisation of the ligand was observed. However, at 37°C, the ^{125}I activity appeared to increase steadily until 30 minutes, after which time a gradual decrease in internalised ligand was seen to occur. Inclusion of the exogenous amine, ammonium chloride, into the experimental set-up, caused the ^{125}I activity to continue increasing throughout the entire timecourse of the experiment. No decrease was observed after 30 minutes as before. This implied that lysosomal degradation may have been the reason for the decrease.

Sucrose gradient fractionation techniques were used to observe the fate of the ligand after internalisation, the rationale being that the various components in the cells would separate out at different densities of sucrose when subjected to a centrifugation step in a linear sucrose gradient. These experiments further indicated that once internalised the ligands made their way to the lysosomal compartment. This was determined from the observation of increased ^{125}I activity in denser fractions of the sucrose gradient known to be associated with the lysosome.

Larger α-MSH analogues were also prepared, iodinated and their binding and internalisation studied in the B16 murine melanoma cell system. As for ^{125}I-[Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH, ammonium chloride was tested in the system. Overall, the larger the ligand, the slower the rate of internalisation.

Overall, the results reported in this thesis strongly favour the concept that α-MSH analogues are internalised into B16 murine melanoma cells via receptor-mediated endocytosis. Moreover, once inside, they make their way to the lysosomal compartment where degradation may occur.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr Colin Pouton, Dr Sarah Branch, Dr Lidia Notarianni and Dr Steve Moss for their constant support and enthusiasm throughout the time on this thesis. I would also to like to thank Dr George Olivier at Bath University for synthesising the peptides employed in this work. I would like to thank my family for their constant support and encouragement, and especially Mr Nick Dorrell for his friendship and understanding throughout. I would also like to thank the SERC/DTI funded LINK programme in 'Selective Drug Delivery and Targeting' for the funding of this work, and also Professor David Davies, Head of School, for providing facilities for the research.

ABBREVIATIONS

ACTH	Adenocorticotropic Hormone
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Mono-Phosphate
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
FCS	Foetal Calf Serum
hCG	Human Chorionic Gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
LDL	Low Density Lipoprotein
LPH	Lipotropin
M-Daunomycin	Melanotropin-Daunomycin
MSH	Melanocyte Stimulating Hormone
PBS	Phosphate Buffer Saline
PMSF	Phenylmethyl-sulphonyl Fluoride
RES	Reticuloendothelial System
TFA	Trifluoroacetic Acid
WGA	Wheat Germ Agglutinin

AMINO ACIDS

Arg	Arginine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Lys	Lysine
Met	Methionine
Nle	Norleucine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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CHAPTER 1

INTRODUCTION

1.1 The Rationale Behind the Concept of Drug Targeting

The selective delivery of chemotherapeutic agents to their site of action should increase therapeutic effectiveness and limit side effects. The clinical use of a therapeutic agent can only be justified if its beneficial effects outweigh its toxic effects (Sezaki, H.; 1984). Most drug therapies currently available, however, exhibit little, if any site specificity. Many times a drug has limited or no access to its intended site of action or is prematurely metabolised or excreted. In other instances, the drug travels rather freely throughout the body and not only acts on the desired target tissues but also causes undesirable effects on non-target tissues (Friend, D.R.; 1987). Most medications are, thus, associated to some degree with toxic side effects. This principle is best illustrated by the use of chemotherapeutic agents in the treatment of cancer where inhibition of tumour growth is invariably accompanied by serious toxicity to rapidly proliferating normal cells. Hence, the need for effective drug delivery systems is straightforward. For a drug to produce a specific pharmacological response it must gain access to its specific "site of action". In the design of specific drugs and drug delivery systems, particular attention must be given to:-

1. The desired site of action of the drug.
2. The route of administration of the drug.
3. The pathway or barriers that the drug must pass *en route* to its destination.

Depending on the nature of the drug one must also ask whether there are specific regions of the body that the drug must avoid either because it is toxic or because it may be rapidly degraded in that region (Pozansky, M.J. & Juliano, R.L.; 1984).

One of the most fundamental barriers to selective drug delivery involves the so called "targeting" problem. Much of the intellectual excitement in the drug delivery area revolves around the concept of directing therapeutic agents to a particular cell population where desirable effects can be achieved without exposing other cell populations where toxic effects occur. The most obvious example of this would be a toxic agent that can discriminate between neoplastic and non-neoplastic cells. However, many other examples related to infectious diseases and genetic disorders come readily to mind (Pozansky, M.J. & Juliano, R.L.; 1984).

A number of difficulties are involved in achieving a desired pharmacological (or other) response at a selected site without undesired interaction at other sites (Gardner, C.R.; 1985). If we consider the problem of targeting a cytotoxic agent to neoplastic but not to normal cells as an example, the following difficulties may need to be overcome.

First, this approach presumes the existence of something to aim at, that is, some molecular characteristic that differs between target and non-target cells. This might be a surface receptor, a structural membrane protein, an intracellular enzyme, or an altered sequence in the genome. Clearly the first task is to establish that a discrete and accessible molecular difference does exist between target and non-target cells. New developments in the characterisation of oncogenes and their products of monoclonal and polyclonal antibodies to tumour associated antigens strongly suggest that it will be possible to establish distinct molecular differences between normal and tumour cells (Eppstein, D.A. & Longenecker, J.P.; 1988).

The second task is to develop a reagent that will show a high degree of selectivity in bringing a toxic drug into (or at least to) the neoplastic cell type. This is far from straightforward in the *in vivo* situation due to involvement in complex sets of non-specific interactions and redistribution.

Finally, one must realise that a neoplastic cell population is not a static entity, but rather a protean, ever changing one. Tumours display an amazing ability to escape or neutralise the actions of drugs or other therapeutic agents to which they were initially sensitive. Some common examples of this are:-

1. The loss of specific receptors.
2. Down regulation of tumour-associated antigens.
3. Shedding of antigens into the body fluids.

Thus targeted drugs which are initially effective may lose their effectiveness as the tumour cell population responds to selective pressures (Pozansky, M.J. & Juliano, R.L.; 1984).

Many efforts have been made to increase the specificity of drugs by association or linkage of the drug to a carrier (Tomlinson, E.; 1987). One major advantage of this approach is that the absorption and distribution of the drug depends on the physio-chemical properties of the carrier, not those of the drug. Thus, the distribution of drugs in the body can be manipulated by alterations in the physico-chemical properties of the carrier and by the choice of carrier.

For the successful delivery of drugs to a specific site via a drug carrier, the design of the drug-carrier complex must conform to a multitude of guidelines:-

1. It must protect the drug from inactivation during transit while minimising premature drug release.

2. It must localise the drug at the site of action, recognise and interact with specific cells and enter the cell or cellular compartment.

3. It must allow for release of the active drug chemically or enzymically at controlled and predictable rates.

4. It must minimise host toxicity and have no adverse side effects.

5. It must be biodegradable, biochemically inert, and nonimmunogenic.

6. It should be easily prepared in a cost-effective, reproducible manner in homogeneous yields.

7. It must be chemically and biochemically stable in its dosage form.

No drug carrier complex has been developed that conforms to all these guidelines. Two types of targeting have been defined - passive targeting and active targeting. The former is a process which utilises the natural (passive) distribution pattern of the drug carrier to deliver drugs to specific sites. This often does not lead to selective uptake by the appropriate target and hence, active targeting has been more extensively studied. Active targeting systems promote selectivity in various ways:- by altering the normal distribution of the drug through use of cell receptors or receptor-recognising molecules; by release of drug only when exposed to specific micro environments such as changes in pH or temperature; or by directing of drug-carrier complexes via magnetic control. Macromolecular and cellular or particulate drug carriers that have been investigated or proposed for site-specific drug delivery - passive or active - include antibodies (Ghose, T.; 1983), glycoproteins (Dean, R.T.; 1979), lipoproteins (Gregoriadis, G.; 1979; Weinstein, J.N.; 1981), lectins (Shier, W.T.; 1979), hormones (Várga, J.M. & Asato, N.; 1983), cells (Zimmermann, U.; 1983), liposomes (Gregoriadis, G.; 1979; Weinstein, J.N.; 1981), DNA (Trouet, A. *et al*; 1979), dextran (Molteni, L.; 1979) and micro- and nanoparticles with or

without magnetic control (Tomlinson, E. & McVie, J.G.; 1983). Various synthetic polymers, such as N-(2-hydroxyypropyl) methyacrylamide copolymers (Duncan, R. *et al*; 1983) and polylysines (Arnold, L.J.; 1983), have also been proposed as components of site-selective drug delivery systems. In addition to prodrugs and drug-carrier complexes, other techniques that have been used to deliver drugs to their site of action include localised chemotherapy whereby the drug is administered directly into the target site, and the use of implantable fusion pumps (Friend, D.R. & Pangburn, S.; 1987).

Before discussing in more detail different drug carriers, an account of the mechanism of entry of macromolecules into cells will first be described.

1.2 Entry of Macromolecules Into Cells - Endocytosis

Macromolecules because of their large size are generally considered to be impermeable to cell membranes and consequently unable to cross transport barriers in biological systems. By virtue of this impermeability, many macromolecules have been successfully used as drug carriers in order to avoid a rapid clearance from the circulation, or to achieve a sustained release when implanted in tissues. Macromolecules used in this type of drug delivery include naturally occurring biopolymers, for example, dextrans, albumin, nucleic acids, and synthetic polymers such as poly(ethyleneglycol). Other macromolecules have a strong affinity to cells due to their multiple binding capacity to either charged or hydrophobic regions on the cell surface. Furthermore, many of the natural molecules such as antibodies and hormones, possess high specificity for binding to selective cells or tissues. These properties can be exploited in the design of carrier-mediated delivery systems that can enhance the absorption or penetration of drugs, particularly, at selective

sites. Examples of the systems just mentioned, and their use in drug delivery to the present day, will be described in detail later.

Due to their impermeability, cellular uptake of macromolecules into mammalian cells depends primarily on a process referred to as endocytosis. In endocytosis, exogenous macromolecules either present in the fluid phase or bound to the cell surface, are first internalised by the cell via the formation of plasma membrane derived vesicles. These vesicles can then be processed through various intracellular organelles and in some cases, recycled to the plasma membrane.

Endocytosis is an extremely complex process involving routing, sorting, fusion of intracellular organelles, modification and recycling of plasma membrane and acidification and degradation of internalised ligands. The extent of endocytosis, for example, can determine the efficiency of protein delivery into target cells.

The following sections will focus on the relevance of endocytosis in the development of drug delivery systems and the diagram on page 7 illustrates the pathway of endocytosis.

Endocytosis refers to the process of internalisation of extracellular particles and fluids by plasma-membrane derived vesicles. The process of internalisation of large particles is referred to as phagocytosis, and, pinocytosis refers to the internalisation of extracellular fluid.

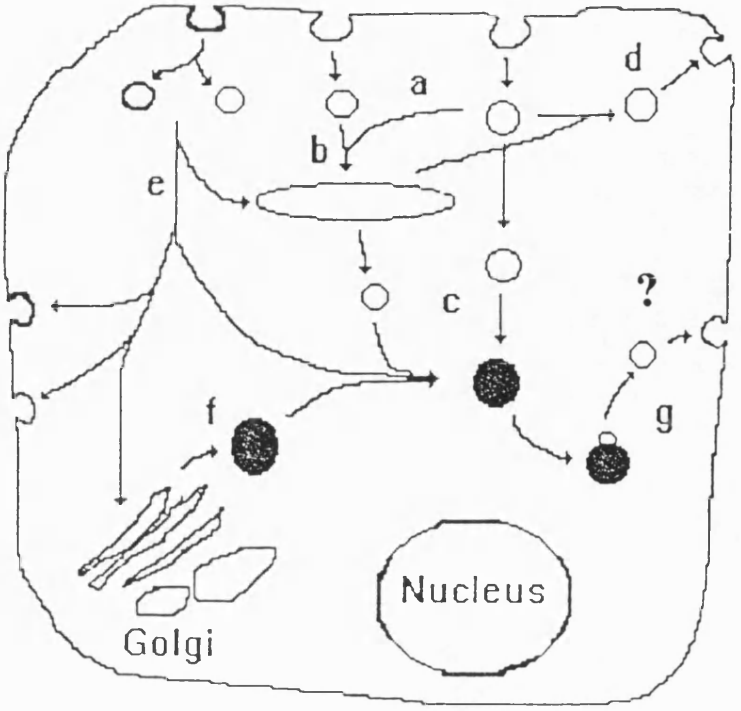


Figure 1.1: A schematic representation of the vesicular pathways of endocytosis. (a) Formation of uncoated vesicles from uncoated pits which may fuse with each other before reaching the early endosomes (b). Vesicles from the endosomes, or directly from invagination of cell membrane, fuse with primary lysosomes to form secondary lysosomes (c). Some vesicles recycle back to the cell membrane (d). Coated regions of the membrane can give rise to vesicles with or without a clathrin coat (e). these either fuse with early endosomes, primary lysosomes, the Golgi complex, or the opposing cell membrane (transcytosis). (f) Vesicles from Golgi complex fuse with primary lysosomes. (g) Vesicles arising from lysosomes may fuse with the plasma membrane.

1.2.1 Receptor-Mediated Endocytosis

Less than 10% of total plasma membrane-associated proteins can have specific affinity for a variety of molecules. These membrane-associated proteins are considered as receptors, and the molecules which specifically bind to these receptors are known as ligands. Following binding on the cell surface the resultant ligand-receptor complex may be internalised. In many cases, internalisation is essential for their physiologic functions.

Two major pathways of receptor-mediated endocytosis have been found to occur, and a brief description will be outlined below:

1. Clathrin-dependent pathway: the formation of ligand-receptor complex is followed by concentration in clathrin-coated regions, or coated pits, of the plasma membrane. These coated pits invaginate from the plasma membranes and turn into coated vesicles by pinching inward from the membrane. The coated vesicles lose their clathrin coats rapidly, and, as a result, smooth membrane vesicles and tubules are formed. These early endosomes, which carry receptors and ligands, subsequently participate in a sequence of intracellular processing and sorting events.

2. Clathrin-independent pathway: this pathway refers to vesicle formation derived from the invagination of non-clathrin-coated plasma membrane. It occurs in receptors with a low population and at a slow rate of internalisation, when compared to that of clathrin-mediated endocytosis described above.

1.2.2 Non-Receptor Mediated Endocytosis

This type of endocytosis occurs when no receptor is involved in the internalisation of the extracellular matter into the cell. It is derived from constant cell membrane internalisation and turnover and is usually unsaturable occurring in both clathrin-dependent and clathrin-independent pathways. Two types of pathway have been discovered for this non-receptor mediated endocytosis:

1. Fluid-phase endocytosis: due to constant turnover of the plasma membrane, small droplets of liquid can be enclosed by an invaginated membrane vesicle and then internalised, dragging whatever solutes happen to be in the droplet. As expected this is a non-saturable process and the majority of internalised contents reach the lysosome where they are catabolised.

2. Adsorptive endocytosis: macromolecules which show a net positive charge or strong hydrophobicity can be adsorbed readily onto the cell surface, and internalised into the cell via membrane invagination eventually going to the lysosome where degradation occurs.

1.2.3 Intracellular Processing of Endocytosed Macromolecules

The intracellular processing of internalised macromolecules involves several compartments in the course of a multi-step transport sequence. They are particularly important when macromolecules are used as drug carriers and an intracellular or transcellular release of active drugs is required for the pharmacological action. In this regard, it is essential to identify the biochemical environment along the endocytotic and transcytotic pathway which can be utilised for the processing of internalised macromolecules.

Endosomes

This is the place where receptors internalised from the cell surface are transferred to. This system which is extremely pleomorphic morphologically, extends from the peripheral cytoplasm, below the plasma membrane to the Golgi area, adjacent to the nucleus. The peripheral elements include vacuoles which are often connected to extensive tubular cisternae, and it is with these elements that vesicles coming in from the plasma membrane fuse. A wide variety of different ligands internalised by receptor-mediated endocytosis have been co-localised within the peripheral elements of the endosome, (Willingham, *et al*, 1981) and it seems probable, therefore, that these elements represent the first intracellular compartment of the endocytic pathway, irrespective of the form of invagination responsible for uptake at the plasma membrane.

The rate at which internalised tracers transfer through the peripheral endosome (5 - 20 minutes) correlates reasonably well with the rate at which recycling receptor populations such as LDL and transferrin are processed intracellularly. It is probable therefore, that these receptor populations can return directly to the plasma membrane from peripheral endosomal elements. The mode of transfer on the return leg of the cycle has not been identified, but is presumed to be a vesicular element. The luminal contents of peripheral endosomal elements have a low pH (5.0 to 5.5), which is largely maintained by an ATP-dependent proton pump located within their limiting membranes. The acid environment within the endosome encourages some receptor-ligand complexes to dissociate and the ligand is released into the lumen, while the unoccupied receptor is free to recycle to the plasma membrane.

Not all internalised ligand complexes dissociate in the acid environment of the peripheral endosome. Those which remain intact in the acidic environment may be routed through the cell to new plasma membrane domains and thus achieve a transcellular transfer.

Lysosomes

This is where most ligands taken up by endocytosis are eventually transferred to. It must be noted that there is evidence that macromolecular materials taken up non-specifically in the fluid phase will reach this degradative compartment but the efficiency of transfer is much less than via receptor-mediated endocytosis.

Lysosomes receive internalised ligands from the peripheral endosome and several recent studies of living cells filmed in culture suggest that the juxtanuclear concentration probably arises largely as a result of endosomal elements migrating inwards from the cell periphery. Tracers introduced into the peripheral endosome system gain access to the juxtanuclear elements after a lag of 15 minutes or more. (Hopkins, C.R.; 1984). Therefore, most internalised physiological ligands in some manner, yet to be determined, gain access to lysosomes. Transfer from endosomes to lysosomes only occurs at temperatures above 22,° C, demonstrating that it is a discontinuous step and suggesting that some form of membrane fusion is required.

1.2.4 Characteristics of endocytosis important for drug delivery.

As the intricacies of the process of receptor-mediated endocytosis unfolded over the last decade, crucial advantages that the process offered to achieve site specific drug delivery began to be apparent and exploited. These advantages accrued from:-

1. High affinity of the receptor for ligand permitting effective sequestration of the ligand from low concentrations in the medium.
2. Rapid recycling of the appropriate receptor molecules permitting building up of relatively high intracellular ligand concentrations.
3. Expression of specific receptors only on certain cell types permitting design of cell type specific drug delivery systems.
4. Specificity as to intracellular compartmentalisation of the ligand depending on the nature of the receptor-ligand duo.

The knowledge-base in the area of receptor-mediated endocytosis is being applied towards the quest for the ideal target drug of the future which should have at least three elements:-

1. An element for specific recognition by the target cell.
2. An element which would elicit the pharmacological action only at the target site.
3. An element designed to facilitate crossing of anatomic barriers to the target cell.

In general, receptor-mediated stratagems for selective drug delivery, of necessity, depend on detailed knowledge about the nature of the ligand, relative distribution of the receptor on various cell types, and the intracellular pathways followed by the receptor-ligand complexes. As a general rule, efficient drug delivery would be possible only through

receptor systems that are recycled rapidly and participate in multiple rounds of drug delivery.

1.3 Controlled Drug Delivery

Now an attempt will be made to critically evaluate a range of different drug carrier systems including lipid structures such as liposomes, denatured albumin microspheres, plasma proteins such as low density lipoproteins and synthetic macromolecules. No attempt will be made to include all drug delivery systems available at present. In addition both systems which have yielded positive results and those where less encouraging data has been obtained will be discussed.

1.3.1 Liposomal Drug Delivery

Liposomes, also known as phospholipid vesicles, have been one of the most popular experimental approaches to controlled drug delivery. Several reviews have been published on this topic including those by Weinstein, J.N.; (1981), but, a brief summary of their chemical and physical characteristics will be included here.

Liposomes are discrete artificial vesicles composed of one or more concentric lipid bilayers enclosing an equal number of aqueous spaces. They range in size from 0.02μm to 100μm. Due to the fact that liposomes can hold drugs within their aqueous spaces, thus protecting the drug from the bioenvironment, they are heralded as the ultimate drug carrier (Friend, D.R. & Pangburn, S.; 1987). However, physical limitations greatly hinder their usefulness in site-specific drug delivery *in vitro*. This is mainly due to their size, an

intravenous load of liposomes is taken up to a large degree by phagocytes of the liver and spleen (Gregoridias,G.; 1979). Other problems include:-

1. They cannot easily leave the circulation to reach extravascular targets due to size effects.
2. Leakage of liposomal contents prior to reaching the target also has been reported.
3. Controlling release of the drug once the liposome reaches the target has proved difficult (Juliano,K.L.; 1982).

The major reason why liposomes have been less successful than originally hoped is the fact that cell fusion appears to be the primary method of introducing liposomal contents into the cytoplasm. However, spontaneous fusion is a low-level process at best; cells do not fuse with each other in nature, and it is overly-optimistic to expect man-made cells to do so. At present, the promotion of fusion or endocytosis, for example, with viral coated proteins, is one way in which sensitive liposomes may lead to future applications that could well promote the usefulness of liposomes in general.

Recently, Woodle, M.C. *et al* (1992) reported that a novel liposome formulation, the stealth (a registered trademark of liposomal Tech.;Inc), with reduced RES uptake and prolonged circulation times had been developed. The new long-circulating liposomes have been used to deliver cytotoxic drugs more selectively to tumours and improve targeting of ligand-bearing liposomes to cells in the vascular system. Their results demonstrated the value of using certain long-circulating liposome formulations to deliver a peptide hormone (vasopressin). Many factors which influence liposomal drug delivery were addressed, including liposome circulation time, hormone release during circulation, blood levels of the "free" peptide required for bioactivity, clearance rate of the free drug, maximum plasma levels that can be tolerated, and the fate of encapsulated peptide cleared with the

liposomes. Although, a detailed characterisation of these parameters is lacking, the potential of such an approach may be applicable to improving the delivery of therapeutic agents used in the treatment of acute disorders which currently require high doses and frequent injections or infusions to maintain therapeutic blood levels (Woodle, M.C. *et al* ; 1992).

1.3.2 Antibodies

Antibodies are believed to hold the most promise of using protein carriers to act as a drug delivery system. The availability of relatively large quantities, through hybridoma technologies, of pure antibodies with defined specificities towards the cell surface antigens has made it possible to target drugs and toxins to particular tissues as well as cell types. The major handicaps of such processes is the inability of significant amounts of the complex to reach the target, and, the shedding of antigens into the general circulation. The fact that antigens produced by cancer cells are constantly changing further complicates the use of antibodies by lowering specificity of antibody-drug conjugates prepared for an earlier form of cancer cell (Suzuta, T.;1983, O'Neill, G.J.; 1979).

Overall, immunotoxins (that is, a monoclonal antibody acting as a carrier for a toxin which is linked via a chemical bond) allow the flexibility of using a wide range of carrier molecules, hence increasing the number of diseases for application. Of recent developments, immunotoxins consisting of CD4, anti-P-glycoprotein, and interleukins have been used selectively to kill HIV-infected cells (Till, M.A. *et al;* 1988), multiple drug resistant (MDR) cells (Fizgerald, D.J. *et al;* 1987), and various cells expressing growth factor receptors (Ogata, M. *et al;* 1988, Chaudhary, Y.K. *et al;* 1987), respectively. Chimeric toxins have also been produced by genetic engineering technology using fused

toxin-carrier genes. This technology to carry out conjugation reactions and reduces the number of purification steps necessary to obtain the pure immunotoxin (Shen, W-C. *et al;* 1992).

1.3.3 Lectins

Lectins are glycoproteins which have the affinity towards specific carbohydrate and sugar residues on the cell surface. Several lectins have been used as carriers to increase the binding and uptake of proteins in mammalian cells. For instance, wheat germ agglutinin (WGA), a plant lectin, binds sialic acid and *N*-acetylglucosamine residues on the cell surface and enters the cells by adsorptive endocytosis. Conjugates of WGA with HRP are endocytosed and subsequently transcytosed across the brain capillary endothelium. The Golgi apparatus has been implicated in the transcytosis of the WGA-HRP conjugates across the blood brain barrier (Sharon, N. & Lis, N.;1989, Broadwell, R.D. *et al;* 1988).

1.3.4 Polylysines

Polylysine, is just one of a number of synthetic polymers which has been used as a carrier of bioactive agents in all facets of drug delivery. The approach to delivering polymeric drugs site specifically has generally followed one of two routes:-

1. The polymeric drug acts as a lysosomotropic agent, and this relies on the concept of lysomotropism to attain specificity; or
2. Homing devices - antibodies, hormones, or receptor-specific sugar residues - are used to concentrate the polymeric drug at its target.

Without a mechanism for concentrating the drug-carrier complex at the target site, there is probably little hope that variations in the endocytic activity of cell type will be adequate to achieve significant site selectivity.

Poly(L-lysine) is a water-soluble, cationic homopolymer of repeating L-lysine units. It has many characteristics that would indicate its usefulness as a drug carrier including:-

1. It has some affinity for negatively charged cancer cells.
2. It appears to be taken up by endocytosis.
3. It is very susceptible to degradation by trypsin and therefore is biodegradable.

Major drawbacks of poly(L-lysine) are biological activity and its toxicity to animals at elevated doses or in high molecular-weight forms.

In vitro experiments whereby poly(L-lysine) has been incorporated as a drug carrier for methotrexate, daunomycin, adriamycin and 6-aminonicotinamide have been attempted. Despite successful inhibition of various tumour cell lines *in vitro*, drug-poly(L-lysine) conjugates have shown little effectiveness when tested *in vivo*. It is apparent that extrapolation of results obtained *in vitro* to events expected *in vivo* can be misleading. Mechanisms for the distribution and metabolism of polymeric drugs are generally missing in most *in vitro* tests. It is therefore not surprising that a poor correlation often exists between *in vitro* and *in vivo* results.

1.3.5 Dextrans

Dextran, a polysaccharide, has been used as a plasma expander but also has been studied as a drug carrier. The advantages of dextran as a drug carrier are:-

1. High water solubility.
2. Well characterised chemical structure.
3. Availability of different molecular weight fractions.
4. Low toxicity.
5. Low pharmacological activity.
6. Protection of conjugated drugs from biodegradation.

Drugs can be coupled to the hydroxyl groups of dextran an a variety of ways including periodate oxidation, cyanogen bromide activation and diazotization.

One of the best documented examples of dextran used as a drug carrier, is its conjugation to mitomycin C. Mitomycin C, injected as the free form, was rapidly cleared from plasma of rats. On the other hand, when conjugated with dextran, mitomycin C was retained for a considerably long period. It must be noted that, even when conjugated to dextran, sustained plasma concentrations of free mitomycin C were obtained. Thus, mitomycin - dextran conjugates are considered to act as a reservoir of mitomycin C which behaves characteristically as a macromolecule while supplying the drug in the body (Sezaki, H. & Hashida, M.; 1984).

1.3.6 Hormones

Hormones, are an additional example of protein carrier used in drug delivery. Since they exert their effect after binding to specific receptors, on their target cells, attempts have been made to deliver drugs to specific cells via hormone carriers. Some hormones investigated as drug or toxin carriers include human placental lactogen (Chang, T.M., *et al*; 1977), human chorionic gonadotropin (Oeltmann, T.N. & Heath, E.C.; 1979), epidermal growth factor (Crowley, D.B.;1980) and melanotropin (Pozansky, M.J. & Olela, L.C.; 1980). Only the latter, melanotropin-drug conjugates, were actually tested *in vivo*, and although *in vitro* results looked promising, the *in vivo* studies resulted in poor efficacy (Varga, J.M. & Asato, N.; 1983).

Daunomycin was linked to melanotropin via the ε-amino groups of lysine and its terminal group, aspartate. DBA mice were injected with 2×10^6 melanoma cells. This was followed by subcutaneous injections of daunomycin-melanotropin conjugate, either concomitantly or one or two weeks later, at a site distant from where the melanoma cells were injected. Most of the animals injected simultaneously with melanoma cells and daunomycin-melanotropin were dead at two weeks.

Reasons for this lack of success include, the possibility that the cutaneous melanocytes were not the only noncancer cells possessing MSH receptors. In addition, endogenous ligands may have been competing for a finite number of receptors on target cells. Alternatively, rapid clearance could have produced such results (Friend, D.R. & Pangburn, S.; 1987).

Although these attempts were unsuccessful, knowledge of a hormone's receptor characteristics, such as cell-type specificity, and whether or not the drug-hormone

conjugate is internalised, may improve the use of hormones in site-specific drug delivery. The section following this takes a more detailed look at the possibility of peptides used in drug targeting.

1.4 Peptide Drug Targeting

As mentioned previously, the purpose of drug targeting is to reduce toxic side effects and increase the therapeutic index. As discussed in section 1.3 above, drugs have been linked to a wide variety of carriers, namely antibodies, glycoproteins, lectins, DNA and liposomes. Few such drug-carrier complexes have reached the stage of clinical trials.

Hormones may offer some advantages compared to other substances for the following reasons:-

1. They are relatively easy to obtain in chemically pure form, due to the ease of synthesis and low expense.
2. They are not bound by Fc receptors on macrophages.
3. Because hormones are structurally identical or very similar in different species, no immunogenic reactions are expected.

The mechanism of action of the polypeptide/hormone is that the hormone binds to the receptor - an essential requirement for hormone action. The chemically well characterised hormone interacts with the poorly characterised receptor, an integral protein of the plasma membrane, in a saturable fashion to form a hormone-receptor complex. The magnitude of the hormone-receptor complex at the cell surface is a major determinant of the magnitude of the response. The concentration of hormone at any given time at the cell surface is a

function of the concentration of the hormone bound and hormone internalised. Similarly, the concentration of receptor is critically determined by the amount of membrane internalised; internalisation, therefore, provides a simple mechanism that couples hormone degradation with receptor regulation. Overall there are several diverse functions of the hormone receptor complex. These include the fact that both membrane and intracellular biological activities are linked in some way to the hormone-receptor complex; degradation of the ligand is linked to the binding of the ligand; and ligand-induced regulation of the receptor is linked to the initial binding process (Gorden, P. *et al*; 1980).

Overall, hormone-receptor interactions are unique in three areas. These are cross-recognition of structurally similar hormones, for example, M-Daunomycin could be recognised by cells carrying ACTH and LPH receptors, since the peptide sequence of melanotropin contains a homologous region with ACTH, LPH and other portions of the untailored stem hormone of the ACTH family (Lowry, P.J.; 1977). Structural homology of hormones need not be the only reason for cross-recognition of the same hormone by different receptors. There are indications that cell surface receptors are not strictly monospecific. The best studied examples are the antigen combining sites for which the 3 dimensional structure of receptor-ligand complexes have already been analysed by X-ray crystallography. In addition, interaction of β-melanotropin with the combining sites of immunoglobulins: 4 out of 10 randomly chosen myeloma proteins were found to bind proteolytic fragments of β-melanotropin. Finally as it follows from the multispecificity of receptors, the frequency of the occurrence of ligand-receptor interactions is a function of binding energy. A ligand may interact with one type of receptor with a relatively high energy of binding (e.g. K_0 $10^9 lmol^{-1}$) or alternatively may bind to a great number of receptors with lower binding energies (K_0 $10^6 lmol^{-1}$). In view of this, it can be clearly

seen that it is essential to take great care when choosing a toxin. For instance, if it only takes a few toxin molecules to be lethal to the cell, then occupation of a few low affinity receptors by the hormone-toxin conjugate may lead to the destruction of a great variety of cell types carrying low affinity receptors for the hormone (Varga, J.M. & Asata, N.; 1983).

The fact that hormones generally exert their effect after binding to specific receptors on their target cells enhances the potential of using them in selective drug targeting. The distribution of the molecules will therefore be restricted to those compartments in the body, which can be accessed by the available transport mechanism for each type of molecule (McMartin, C.; 1988). Examples where toxins have been coupled to naturally occurring peptides and their therapeutic usefulness studied are summarised below:-

1. Ricin. This has been linked to the cell-specific subunit of human chorionic gonadotropin (hCG). However, its effect was found to be less efficient than for free ricin itself.

2. Diphtheria toxin. This was conjugated via disulphide linkages to human placental lactogen. Although the conjugate was bound effectively, the receptor was unable to mediate the entry of the toxin A fragment.

Both examples emphasise the point that it is not enough for the hormone to bind effectively to its cell surface receptor if internalisation does not occur. Bearing this in mind, selection of a hormone as a vector in site-specific drug delivery requires knowledge of its receptor characteristics, such as cell type specificity, and whether or not the drug-hormone conjugate is internalised. One such hormone which has been studied extensively is MSH (α-melanocyte stimulating hormone), and a brief description of it will now be carried out since it, and its analogues form the basis of this study.

1.4.1 Melanocyte Stimulating Hormone

α-MSH is a pituitary tridecapeptide,

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Figure 1.2: Structure of α-MSH

and is one of several chemically and biologically related peptides which may be derived from a large molecular weight precursor protein, pro-opiomelanocortin, in the vertebrate pituitary and hypothalamus (Sawyer, T.K.; 1982). The site of interaction of the hormone with the cell is a specific receptor localised on the plasma membrane. Secretion of the hormone by the pituitary is under control of the hypothalamus and is subject to circadian rhythm. Various peripheral tissues are MSH sensitive, primarily the skin, where melanocytes are sited. Circulating hormone is mainly inactivated by the skin and skeletal muscles as well as the kidneys, liver, lung and intestines. The inactivation is due to enzymatic cleavage primarily by an endopeptidase at Phe^7-Arg^8 in the sequence, followed by exopeptidase causing degradation to free amino acids. The peptide is also synthesised and secreted within the brain, it is considered a neuropeptide with various CNS related effects on development, adaptive behaviour, learning, neurotransmission and nerve regeneration (Eberle, A; 1988; Leiba, H. *et al;* 1990).

Distribution of the native peptide is not well understood. However when a radiolabelled MSH analogue was injected into B16 melanoma bearing mice, initial uptake occurred in the kidney, spleen, tumour and adrenals. No preference was displayed for the tumour and transport across the blood-brain barrier was minimal Bolognia, J. *et al*; (1989).

The primary effect of MSH in the pituitary is related to skin darkening, it stimulates melanogenesis, proliferation and differentiation of melanocytes. The complex process of skin pigmentation although well defined in other species, for example, amphibia, is less well defined in man. Skin darkening is basically caused by cell melanisation by melanin involving the enzymatic oxidation of tyrosine to melanin (Aroca, P. *et al;* 1989; Jara, J. *et al;* 1989).

The site of interaction of the hormone with the cell is a specific receptor localised on the plasma membrane. MSH binding and subsequent signal transduction appears to require extracellular calcium. Binding stimulates adenylate cyclase causing intracellular levels of cAMP to rise, this in turn activates protein kinases and causes protein phosphorylation (Eberle, A; 1988). It is unknown how the signal caused by MSH binding is terminated, but one or more of three possibilities is thought to occur:-

1. Dissociation of MSH from the receptor.
2. Internalisation of the receptor-ligand complex.
3. Inactivation of MSH.

Although at least part of the receptor/ligand complex is known to be internalised, its intracellular pathway and localisation is not defined as yet (Panasci, P. *et al;* 1987).

Recent studies have provided significant improvements over earlier systems for radioligand binding, although most analogues of α-MSH used still possess technical limitations, such as, relatively low specific activity with subsequent uncertainty in determinations of biological activity, lack of demonstration of biological activity in mammalian cells, and susceptibility to metabolic degradation. Sawyer and his colleagues

have developed a superpotent and enzymatically resistant α-MSH analogue, namely, [Nle^4,D-Phe^7]α-MSH (Sawyer, T.K. *et al;* 1980). They demonstrated unique biological properties including, prolonged biological activity, enhanced potency relative to α-MSH in a number of biological systems, and resistance to degradation by serum enzymes (Tatro, J.B., *et al;* 1990).

Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Figure 1.3: Structure of [Nle^4, D-Phe^7]α-MSH

The use of α-MSH and its analogue, [Nle^4, D-Phe^7]α-MSH as drug carriers have several advantages in addition to their targeting properties. These include the ease of obtaining them in a pure form, the small size which avoids the problems associated with larger conjugates, the ability to study chemical manipulations in the peptide sequence and possible low immunogenicity due to their structural similarity. However, to understand the full potential in selective drug delivery, the binding of the analogues to the α-MSH receptor, followed by internalisation and subsequent fate of the ligands must be examined in detail. This is the major aim of the study presented here. The analogue primarily used is [Nle^4, D-Phe^7]α-MSH and the cellular system involved, is the B16 murine melanoma cell.

1.4.2 The fate of [Nle^4,D-Phe^7]α-MSH after binding to the α-MSH receptor

As was discussed in the preceding two sections, it has been proven that binding of a peptide to its receptor is not enough when targeting of drugs is concerned using a lysosomotropic system. The ligand must be internalised to the cell in order to be effective. It will therefore be a very necessary part of this study to monitor the trafficking of [Nle^4,D-Phe^7]α-MSH in B16 melanoma cells. No work has been reported on such a system, and hence, the research carried out regarding this area could prove invaluable whenever α-MSH analogues are considered as drug targeters. It is essential to have information regarding peptide ligand uptake for such a process. If it is found that internalisation results in movement of the ligand to the lysosomal compartment, this will be of great use as far as drug choice is concerned for lysosomotropic system.

The method by which individual cellular components can be characterised, involves the process known as cell fractionation. Such a process, can be used to dissect the endocytic pathway in the respect that radioligands can be monitored in the various fractions of the cell representing the different cellular components. For instance, if upon cell binding, [Nle^4,D-Phe^7]α-MSH is internalised and makes its way along the endocytic pathway to the lysosome, then the fractions containing the lysosomes should also contain the radiolabelled ligand. Each component of the cell can be identified using enzyme assays specific for that component. For example, the plasma membrane may be characterised by a number of enzymes including, Na^+K^+ATPase, adenylate cyclase and alkaline phosphodiesterase I, to name but a few. On the other hand, lysosomes are identified by acid phosphatase, β-glucuronidase and β-hexosaminidase.

1.5 Overview

In order to assess the binding, internalisation and fate of [Nle^4,D-Phe^7]α-MSH, it is first necessary to confirm that internalisation does occur, and, is dependent upon the α-MSH receptor. Investigations to be carried out include cellular binding experiments both at 4°C and metabolic temperature, 37°C, to determine internalisation of the ligand. If such a process is found to occur, further studies regarding the fate of the ligand after it enters the cell will be performed.

Finally, a study regarding size of the ligand and its effect on the rate of internalisation will be carried out. The same basic analogue, [Nle^4,D-Phe^7] α-MSH will be employed, however, larger molecular moieties will be added to it, and their effect studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Culture Reagents and Buffers

RPMI 1640 + FCS

The media used for the growth of the B16 melanoma cells was RPMI 1640. Table 2.1 lists the individual components used in its preparation, and the various quantities required of each reagent. The media was prepared in an aseptic manner.

REAGENT	VOLUME(MLS)	SUPPLIER
DOUBLE DISTILLED DEIONISED WATER	450	N/A
RPMI 1640	50	IMPERIAL LAB (UK) LTD.
FOETAL CALF SERUM	50	GIBCO
7.5% $NaHCO_3$	13.5	FISONS
MEM NONESSENTIAL AMINO ACIDS	5	IMPERIAL LAB (UK) LTD.
PENICILLIN / STREPTOMYCIN	5	IMPERIAL LAB (UK) LTD.
L-GLUTAMINE	5	IMPERIAL LAB (UK) LTD.

Table 2.1: Formula of RPMI 1640 + FCS medium.

Preparation of $NaHCO_3$ (7.5%)

75g of $NaHCO_3$ was weighed and dissolved in 1000mls of double distilled deionised water. Volumes of 100mls were transferred to 100ml tissue culture glass bottles and sterilised by autoclaving for 15 minutes at 121°C in a Drayton Castle Laboratory Steriliser. The buffer was stored at room temperature and was prepared fresh on a monthly basis.

Other Reagents

All other reagents were used directly as obtained from the supplier, however, the following storage protocols were followed.

Foetal Calf Serum: This was received in 500ml bottles from Gibco and was aliquoted into 100ml samples and stored at or below -20°C.

Penicillin / Streptomycin: This was received in 100ml bottles from Imperial Lab (UK) Ltd. and was aliquoted into 5ml samples and stored at or below -20°C.

L-Glutamine: as for penicillin / streptomycin.

SERUM FREE RPMI 1640

This was prepared in the same way as RPMI 1640 + FCS, however, no foetal calf serum was added and the volume replaced by an extra 50 mls of double distilled deionised water. This was normally used for washing the cells after ligand binding. When prepared it was stored at 4°C.

PHOSPHATE BUFFERED SALINE

One PBS tablet supplied by Dulbecco A was dissolved in 100mls of double distilled deionised water and sterilised. This was prepared on a monthly basis and stored at room temperature.

0.02% EDTA

0.1g of EDTA was dissolved in 500mls of double distilled deionised water containing 5 PBS tablets. After sterilisation by autoclaving, 20 ml samples were transferred to glass universal bottles and stored at or below -20°C for no longer than one month.

20mM NH_4Cl

A 10 times concentrated solution of NH_4Cl (200mM) was prepared by dissolving 10.70g NH_4Cl in 1 litre of double distilled deionised water. A 1 in 10 dilution was achieved by adding 50mls to 450mls serum free medium to give a working concentration of 20mM NH_4Cl.

LEUPEPTIN

A 100 times concentrated solution of leupeptin ($1 mg ml^{-1}$) was prepared by dissolving 1mg of leupeptin in double distilled deionised water. A 1 in 100 dilution was achieved by adding 1ml to 100mls of serum free medium to give a working concentration of $10 \mu g ml^{-1}$ leupeptin.

PEPSTATIN A

A 100 times concentrated solution of pepstatin A ($1mgml^{-1}$) was prepared by dissolving 1mg of pepstatin A in 1ml of methanol. A 1 in 100 dilution was acheived by adding 1ml to 100mls of serum free medium to give a working concentration of $10\mu gml^{-1}$.

PMSF

A stock solution of 200mM PMSF was prepared in ethanol, aliquoted into screw top eppendorffs (100µl) and stored at or below -20°C for up to 9 months. A working concentration of 1mM was acheived by a 1 in 200 dilution in homogenisation buffer.

1mM EDTA

0.037g of EDTA was dissolved in 100mls double distilled deionised water. Aliquots of 20mls were transferred to glass universals and stored at or below -20°C.

2.1.2 Binding Buffer

The table below indicates the reagents included in the binding buffer:

REAGENTS	STOCK CONC.	WORKING CONC.	SUPPLIER
HEPES	250mM	25mM	FISONS
BSA	2%	0.2%	SIGMA
1,10-PHENANTHROLINE	0.13M	0.013M	SIGMA

Table 2.2: Formula for the binding buffer.

Preparation of Stock Solutions

HEPES

6g of HEPES was dissolved in 100mls of serum free medium and buffered to pH 7.4 with 1M NaOH. Aliquots of 5mls were stored in 5ml bijou bottles at or below -20°C and prepared on a monthly basis.

BSA

2g of BSA were dissolved in 100mls of serum free medium. Aliquots of 5mls were stored in 5ml bijou bottles at or below -20°C and prepared on a monthly basis.

1,10-Phenanthroline

0.3g of 1,10-phenanthroline was dissolved in 10 mls of 95% ethanol and stored at or below -20°C.

Preparation of Binding Buffer

5mls of HEPES stock solution, 5mls of BSA stock solution and 5µl of stock 1,10-phenanthroline was made up to 50mls with serum free medium in a grade A volumetric flask. This was prepared directly before use.

2.1.3 Homogenisation Buffer

Four buffers were tested and the formulae for all four outlined Table 2.3:

BUFFER	REAGENTS	CONCENTRATION	SUPPLIER
1	$NaHCO_3$	1mM	SIGMA
	DTT	1mM	SIGMA
2	$NaHCO_3$	1mM	SIGMA
	DTT	1mM	SIGMA
	sucrose	0.25M	FISONS
3	Tris-HCl	0.05M	FISONS
4	Tris-HCl	0.05M	FISONS
	sucrose	0.25M	FISONS

Table 2.3: Formulae for homogenisation buffers tested.

Preparation of buffers

Buffer 1

0.0084g of $NaHCO_3$ and 0.0154g of DTT were dissolved in 100mls of double distilled deionised water.

Buffer 2

0.0084g of $NaHCO_3$, 0.0154g of DTT and 9mls of 66% sucrose were dissolved in and made up to 100mls with double distilled deionised water.

Buffer 3.

0.6g of Tris base was dissolved in 60mls of double distilled deionised water, buffered to pH 7.2 with 1M HCl and then made up to 100mls with double distilled deionised water.

Buffer 4

0.6g of Tris base was dissolved in 60mls of double distilled deionised water, buffered to pH 7.2 with 1M HCl, 9mls of 66% sucrose added and the whole mixture made up to 100mls with double distilled deionised water.

All buffers were prepared on a monthly basis and stored at 4°C.

2.1.4 Acid Wash Buffer

A 0.1M citrate buffer was chosen as the buffer for the acid treatment of the cells after binding experiments had been performed. It was prepared as follows:-

21.01g of citric acid (Fisons), 5.43g NaCl and 23.44mls of 1M NaOH were made up to 1 litre with double distilled deionised water. This formula give the required pH of 2.5, adequate for the removal of externally bound ligand from the surface of the cells. The buffer was stored at 4 °C and prepared on a monthly basis.

Note a series of buffers of varying pH's (pH2 to pH 5) were prepared to determine the optimum pH for removal of extracellularly/surface bound ligand. Figure 2.1 on page 35 illustrates the effect of different pH on the efficiency of removing ligand extracellularly bound for a series of cell well experiments where the level of total binding at 37°C was approximately 11,000 counts per minute.

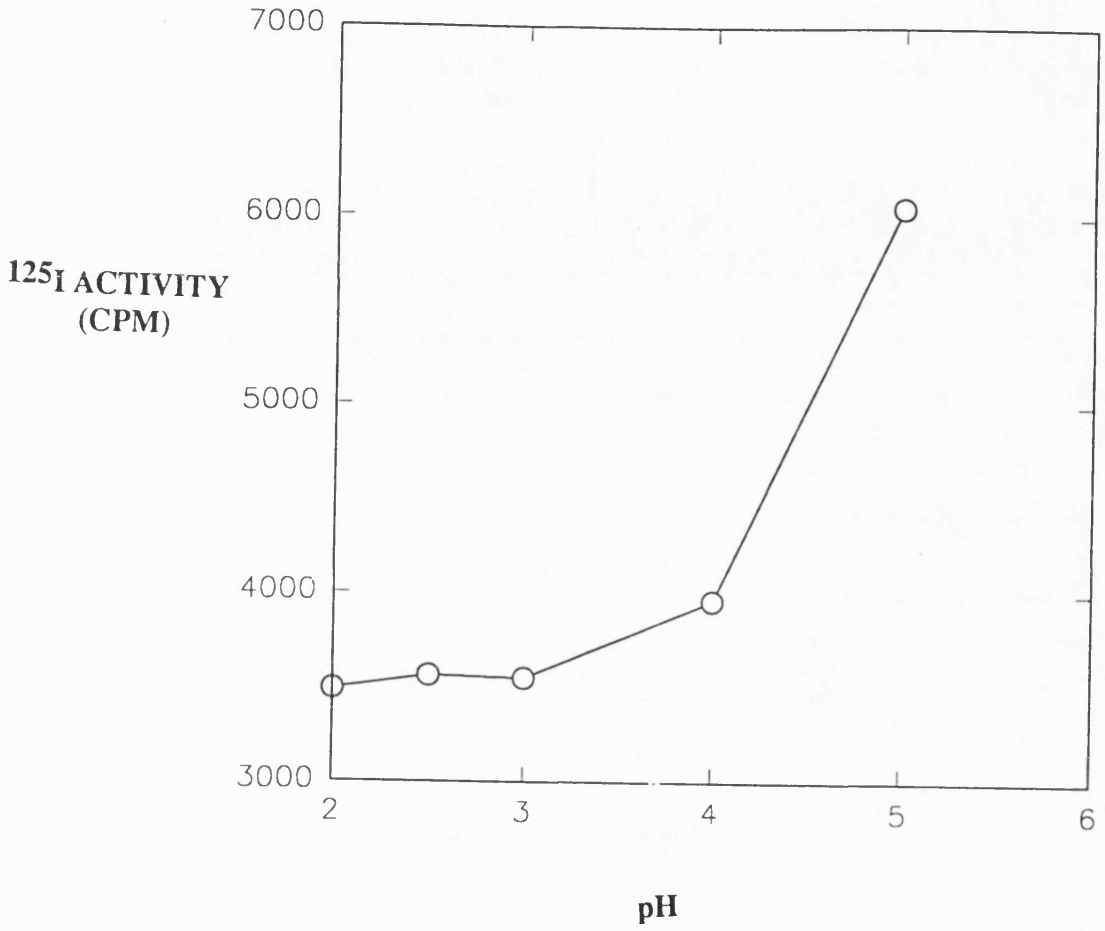


Figure 2.1: The effect of different pH's on the efficiency of removal of extracellularly bound ligand.

2.1.5 Sucrose Gradient Buffers

A 66 % (w/w) sucrose stock solution was prepared by dissolving 171g of sucrose in 90mls double distilled water. The required concentrations of 60% and 10% were then prepared as follows.

60%: 89mls of 66% sucrose + 11mls Tris-HCl

10%: 11mls of 66% sucrose + 89mls Tris-HCl

These were stored at 4°C and prepared on a monthly basis.

2.1.6 Ligands

The following ligands were used, and all were synthesised, purified and aliquoted by Dr G W J Olivier in our own laboratory.

1) Ac-[Nle^4,D-Phe^7]α-MSH.

2) N^{α}-Biotin-[Nle^4,D-Phe^7]α-MSH.

3) Streptavidin Biotin-[Nle^4,D-Phe^7]α-MSH.

Each was prepared as a $1mgml^{-1}$ solution, whereby 1mg of ligand was dissolved in 1ml of sterile 1mM HCl and stored in 100µl aliquots in screw top eppendorffs at 4°C.

2.1.7 Radioisotopes

Reagents

0.25M Phosphate Buffer, pH 7.4

This was prepared from a mixture of 0.25M Na_2HPO_4 and 0.25M NaH_2PO_4

Na_2HPO_4: 4.45g was dissolved in 100 mls of double distilled deionised water.

NaH_2PO_4: 3.90g was dissolved in 100mls of double distilled deionised water.

A pH of 7.4 was achieved by adding NaH_2PO_4 dropwise to Na_2HPO_4. The stock solutions were stored at 4°C and prepared on a monthly basis. The phosphate buffer was prepared on the day of the iodination.

0.25% BSA

0.0125g of BSA (Sigma) was dissolved in 1ml of phosphate buffer, pH 7.4 and 4mls double distilled deionised water. This was prepared on the day of iodination.

0.1% Chloramine T

0.01g of chloramine T was dissolved in 10mls of double distilled deionised water directly prior to use.

1% Polypep (Sigma)

0.05g of polypep was dissolved in 1ml phosphate buffer, pH 7.4 and 4mls of double distilled deionised water directly before use.

1% TFA

1ml of high purity TFA was diluted to 100mls with double distilled deionised water and stored at 4°C.

80% MeOH / 1% TFA

80mls of methanol and 1ml TFA were diluted to 100mls with double distilled deionised water and stored at 4°C.

60% MeOH / 1% TFA

60mls of methanol and 1ml of TFA were diluted to 100mls with double distilled deionised water and stored at 4°C.

50% MeOH / 1% TFA

50mls of methanol and 1ml of TFA were diluted to 100mls using double distilled deionised water and stored at 4°C.

2.1.8 HPLC Solvents

Two mobile phases were required for the purification of radiolabelled ligands and were prepared as follows:-

Mobile Phase A: 0.1% TFA / H_2O

This was prepared by adding 0.5mls of TFA to 499.5mls double distilled water (HPLC grade). The solvent was degassed thoroughly and prepared as required.

Mobile Phase B: 70% acetonitrile / 1% TFA / H_2O

350mls acetonitrile, 0.5mls TFA and 149.5mls double distilled water (HPLC grade) were mixed together. The solvent was degassed thoroughly prior to use and was prepared as required.

2.1.9 Enzyme Assays

β-Hexosaminidase

Reagents

4mM *p*-nitrophenyl α -*N*-acetylglucosamine, in sodium citrate-phosphate buffer (0.1M with respect to phosphate), pH 4.5, stored frozen.
0.15M NaCl, stored at room temperature.

50mM NaOH, stored at room temperature.

Alkaline Phosphodiesterase I

Reagents

5 mM Na-*p*-nitrophenol 5' -thymidylate, stored frozen.

0.1M Tris-HCl, pH 9.0, stored at 4°C.

0.5M glycine, 0.5M Na_2CO_3, stored at 4°C.

2.2 METHODS

2.2.1 Procedure for Radioiodination of Ligands

Column Preparation

A Bond Elut, C18 reverse-phase column, spherisorb ODS (Analytical International) was used to carry out the separation of iodinated ligands. The column had to be prepared before use by a series of washes as outlined below.

Wash 1: 3 × 1ml wash with 1% TFA

Wash 2: 3 × 1ml wash with 80% MeOH / 1% TFA

Wash 3: 1 × 1ml wash with 1% polypep

Wash 4: 3 × 1ml wash with 80% MeOH / 1% TFA

Wash 5: 3 × 1ml wash with 1% TFA

Care was taken with the final 1ml 1% TFA wash to ensure a small volume was left on top of the column to avoid the column from "drying out".

Iodination of Ac-[Nle^4,D-Phe^7]α-MSH

In a 1.5ml eppendorff, 1.5 µl of 1mgml^{-1} [Nle^4,D-Phe^7]α-MSH was added to 20µl of 0.25M phosphate buffer, pH 7.4. 1mCi of ^{125}INa was then added. The reaction was initiated by addition of 10µl 0.1% chloramine T and allowed to proceed for exactly 30 seconds. By adding 0.60mls of 0.25% BSA, the reaction was terminated (Olivier, G.J.W.; unpublished results).

The reaction mixture was then transferred to the Bond Elut column, already prepared by the method described above. To separate the free iodide, mono-iodinated [Nle^4,D-Phe^7]α-MSH and di-iodinated [Nle^4,D-Phe^7]α-MSH, to some extent, the following washes were performed.

Wash 1: 2 × 1ml wash with 0.25M phosphate buffer, pH 7.4

Wash 2: 4 × 1ml wash with 50% MeOH / 1% TFA

Wash 3: 2 × 1ml wash with 60% MeOH / 1% TFA

The first 2mls of wash, i.e. phosphate buffer washing, were discarded. The remaining 6mls were further purified to obtain a pure fraction of mono-iodinated [Nle^4,D-Phe^7]α-MSH, the procedure for which is outlined below.

The whole reaction was carried out with great care behind lead blocks in a fume cupboard.

Purification of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH

A pure sample of mono-iodinated ligand was prepared using reverse-phase high performance liquid chromatography. The details of the equipment used was as follows:

. Milton Roy CM4000 multiple solvent delivery system with a 1ml loop

. LDC Analytical Spectromonitor 5000 photodiode array detector, λ=270nm; aufs range 0.01

. Pharmacia FRAC-100 fraction collector

. Servogor 220 chart recorder; chart speed 12cm/h; 10mV

. HPLC column used was reverse phase C18, ODS, 20cm

The ^{125}I mixture was injected in 12 × 0.5ml aliquots onto the HPLC. The following gradient was performed in order to separate the free iodide, mono- and di-iodinated peptide:

TIME (mins)	SOLVENT A (%)	SOLVENT B (%)	EXPONENTIAL
0	95	5	1
5	95	5	1
50	40	60	0.5
55	40	60	0.5
60	95	5	1

Table 2.4: Gradient Method for the HPLC purification of the iodinated ligands.

1ml fractions were automatically collected between 25 and 40 minutes in LP4 gamma-counter tubes. The ^{125}I activity associated with each 1ml fraction was determined by transferring the tubes to the LKB Wallac 1277 Gammamaster automatic gamma-counter and each sample assayed for 2 minutes.

A specimen HPLC trace of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH purification and a trace of ^{125}I activity associated with the fractions collected from the HPLC are shown on the following two pages, figures 2.2 and 2.3. respectively. From previous studies carried out on "cold" iodinations of the ligand, it was found that the mono-iodinated ligand was eluted around 33 & 34 minutes. From the trace, the second series of high ^{125}I activity was probably due to di-iodinated ligand. The mono-iodinated ligand was used in all experiments.

It must be noted that each time an experiment was carried out, a "fresh" iodination and purification was performed. In addition carrier free Na^{125}I containing 80.5×10^{12} degradations per second per milliatom was used to iodinate the ligands. The gamma-counter was approximately 70% efficient and was assumed to be invariant. Thus the values of cpm plotted are proportional to the number of moles bound.

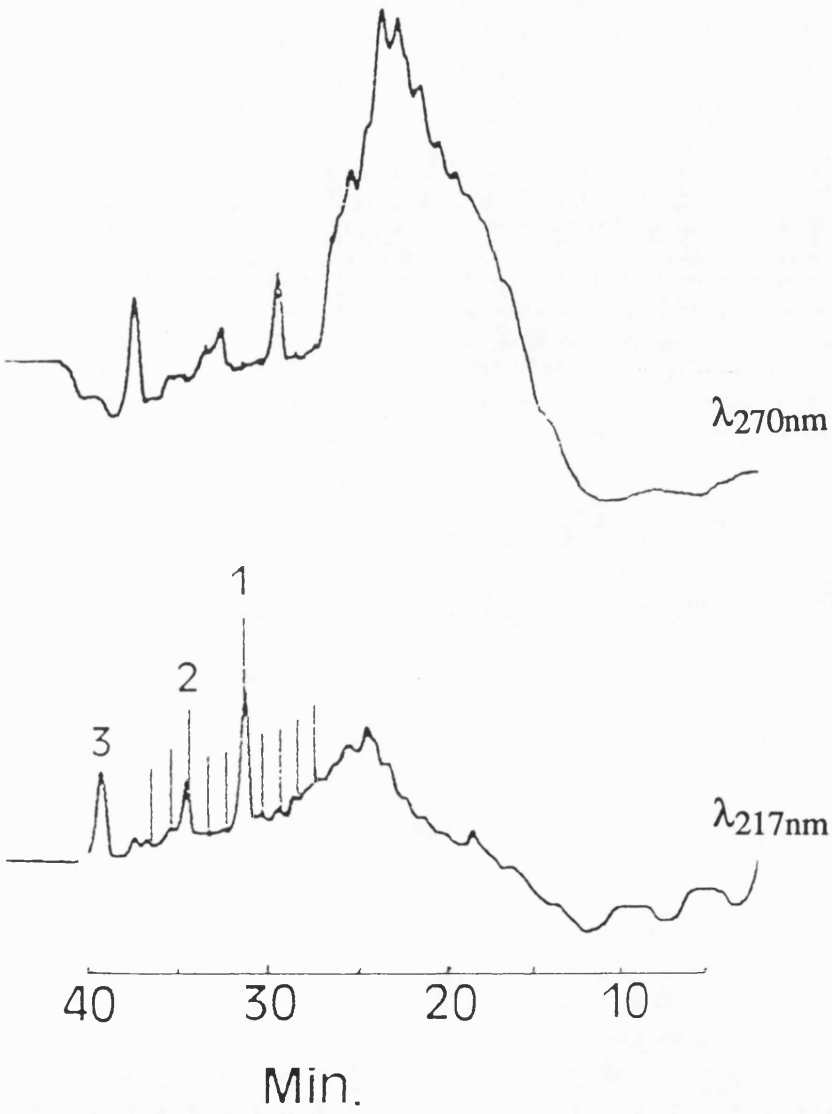


Figure 2.2: HPLC trace of the Purification of Iodinated Ac-[Nle^4,D-Phe^7]α-MSH as measured by UV absorption at 217nm. Peaks 1, 2 and 3 are thought to represent non-labelled, mono-iodinated and di-iodinated peptide respectively.

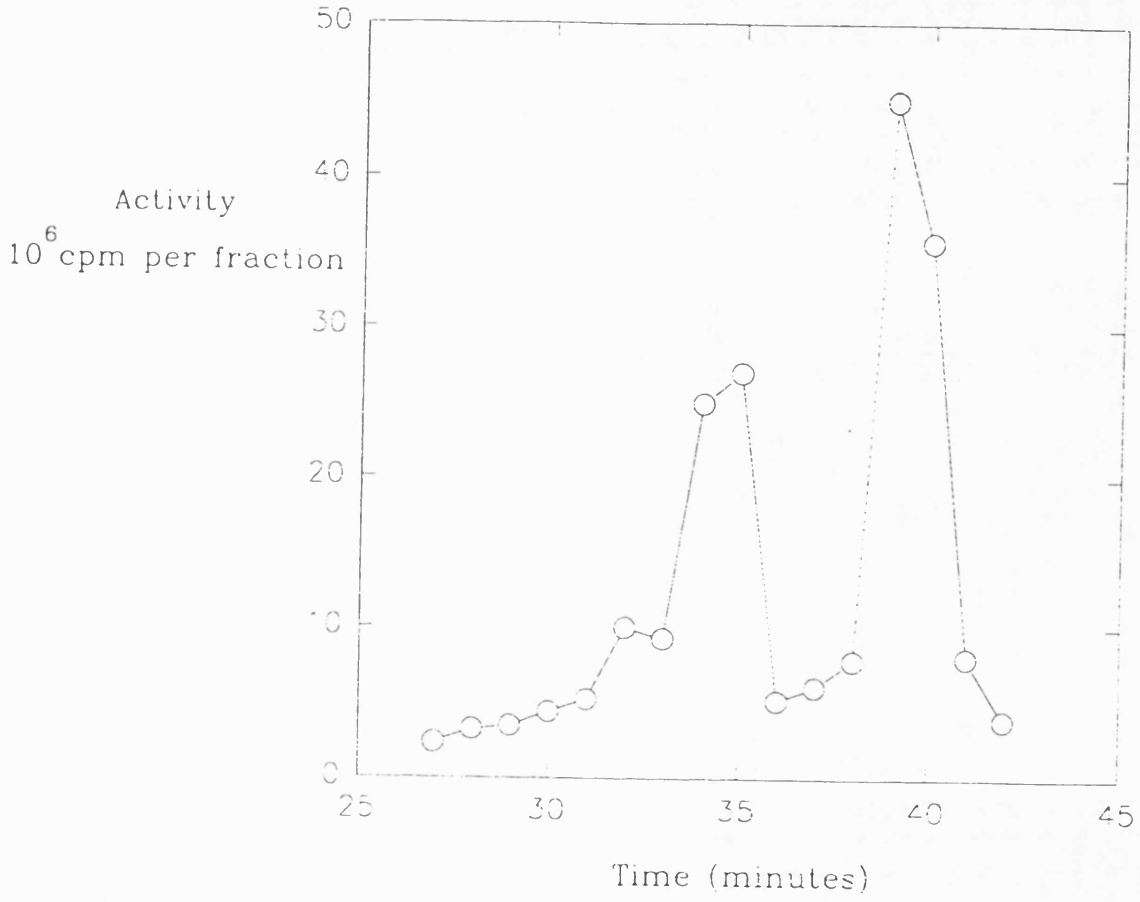


Figure 2.3: Radioactivity Associated with Fractions from the HPLC Purification of Radio-iodinated Ac-[Nle4,D-Phe7]α-MSH from 27 minutes after the Injection sample (Flow Rate 1ml/minute).

Iodination & Purification of N^{α}-Biotin-[Nle^4,D-Phe^7]α-MSH

This was as for [Nle^4,D-Phe^7]α-MSH just described, however, a $1mgml^{-1}$ solution of N^{α} - biotin-[Nle^4,D-Phe^7]α-MSH was used. The fractions collected were between 25 and 42 minutes.

Iodination of Streptavidin-Biotin-[Nle^4,D-Phe^7]α-MSH

The N^{α}-biotin-[Nle^4,D-Phe^7]α-MSH was iodinated and purified as normal (as described above), and the streptavidin added after the iodination as outlined in the next section.

2.2.2 Preparation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α -MSH

N^{α}-Biotin-[Nle^4,D-Phe^7]α-MSH was iodinated and purified in the normal way. The streptavidin was then added using the following procedure:-

Stock streptavidin was 1.66×10^{-5} M ($1mgml^{-1}$) and the required amount was diluted in PBS and added to the purified fraction of N^{α}-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH (Note the required amount depended on the activity of iodinated ligand available). The mixture was placed on an automatic shaker for 30 minutes at room temperature. At the end of the shaking time, the mixture was added to streptavidin coated magnetic beads and left for 10 minutes so as to equilibrate the beads to the magnet. The resulting supernatant should contain the streptavidin-biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH and this was again counted in the gamma-counter to determine the total counts associated. This method was found to yield approimately 85-90% efficiency.

2.2.3 *In Vitro* Experiments

Routine Cell Culture

B16 murine melanoma cells were grown in 175cm^2 Falcon tissue culture flasks in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2mM L-glutamine, 1% MEM nonessential amino acids, 50UIml^{-1} penicillin and 50mgml^{-1} streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Confluent cells were detached weekly with an incubation of 2.5mls of 0.02% EDTA in phosphate buffered saline, pH 7.4, and counted on a haemocytometer. This procedure ensured the cells were at the same stage of growth for each experiment.

Cell Counting and Viability

Once the cells were detached from their flasks, they were then counted for viability. The following procedure was used.

1) 0.4mls of cell suspension was transferred to a test-tube.
2) 0.1ml of 0.1% Trypan blue dye was added and the mixture shaken thoroughly.
3) A small amount of the mixture was transferred to the haemocytometer chamber and , the number of cells in 5 chambers(A-E), as indicated in the diagram below, counted.

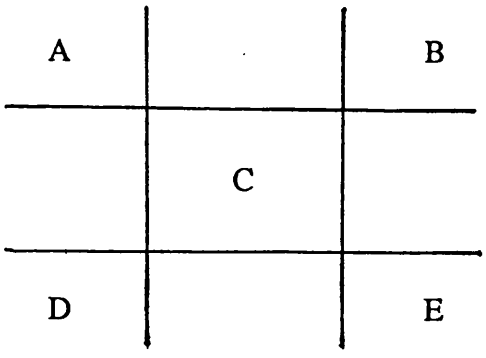


Figure 2.3: Haemocytometer chamber used for Cell Counting

The number of cells per ml was calculated using the following equation:

$$\text{cells / ml} = \frac{(\text{total cells in all 5 chambers}) \times 10^4}{4}$$

The cells not containing the blue dye are the viable cells containing intact membranes, and only those were counted.

2.2.3.1 Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C

A density of 5×10^5 cells per well were incubated in 24-well culture plates for 24 hours in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 1% MEM nonessential amino acids, 2mM L-glutamine, 50IUml^{-1} penicillin and 50μgml^{-1} streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

They were then washed twice with ice-cold serum free RPMI 1640 medium to remove all serum proteins. The cells were then incubated with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH in binding buffer (25mM HEPES, pH 7.2, 0.2% BSA, 0.013mM 1,10-phenanthroline) in the absence or presence of excess non-iodinated [Nle^4,D-Phe^7]α-MSH (1μM) for various times at 37°C. At the end of the required time the binding buffer was discarded and the cells washed with ice-cold serum free RPMI 1640 medium. The ^{125}I activity associated with the cells was determined by digesting the cells with 0.5mls 0.1M NaOH and assayed using the LKB Wallac 1277 Gammamaster automatic gamma-counter. In parallel to this some cells were treated with a 5 minute acid wash at 4°C using 0.1M citrate buffer, pH 2.5. The cells were then removed from the wells using NaOH digestion and assayed for ^{125}I activity in the automatic gamma-counter.

2.2.3.2 Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 4°C

This was carried out to confirm that internalisation only occurred at 37°C. The exact same protocol as for "Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C" was used, except all incubations took place at 4°C.

2.2.3.3 Effect of Ammonium Chloride on the Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

The same protocol as for "Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C was used, however, the cells were incubated for 15 minutes at 37°C in serum free RPMI 1640 medium containing 20mM NH_4Cl prior to incubation with radiolabelled ligand. Thereafter all buffers contained 20mM NH_4Cl.

2.2.3.4 Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

A density of 5×10^5 cells were incubated in 24-well culture plates for 24 hours in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 1% MEM nonessential amino acids, 2mM L-glutamine, 50IUml^{-1} penicillin and 50μgml^{-1} streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

They were then washed 2 times with ice-cold serum free RPMI 1640 medium to remove all the serum proteins. The cells were then incubated with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7] α-MSH in binding buffer (25mM HEPES, pH 7.2, 0.2% BSA and 0.013M 1,10-phenanthroline) in the presence or absence of 1000-fold excess non-iodinated ligand for 2 hours at 4°C. At the end of the 2 hour period the cells were washed with ice-cold serum

free RPMI 1640 medium and the cells subjected to a second incubation at 37°C for varying amounts of time. At the end of the required time, the cells were washed with ice-cold serum free RPMI 1640 medium and digested with 0.1M NaOH and the ^{125}I activity associated with the cells determined using a LKB Wallac 1277 Gammamaster automatic gamma-counter. A parallel series of cells were given a 5 minute acid treatment with 0.1M citrate buffer, pH 2.5, at 4°C, washed and digested and assayed as before.

2.2.3.5 Effect of Ammonium Chloride on the Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

The same protocol as for "Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH" was used, however, the cells were incubated for 15 minutes at 37°C in serum free RPMI 1640 medium containing 20mM NH_4Cl prior to incubation with radio-labelled ligand. Thereafter all buffers contained 20mM NH_4Cl.

2.2.3.6 Effect of Leupeptin on the Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

The same protocol as for the "Ammonium Chloride Effect on the Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH" was used, except the 20mM NH_4Cl was replaced with 10μgml^{-1} leupeptin.

2.2.3.7 Effect of Pepstatin A on the Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

The same protocol as for the "Ammonium Chloride Effect on the Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH" was used, except the 20mM NH_4Cl was replaced with 10μgml^{-1} pepstatin A.

2.2.3.8 Pulse Chase Internalisation of Nα-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7] α-MSH

The same protocol as for the "Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH", section 2.2.3.4., however the 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was substituted by 0.1nM Nα- Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH.

2.2.3.9 Pulse Chase Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH

The same protocol as for the "Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH" was used, however the 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was substituted by 0.1nM Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH.

2.2.4 Sucrose Gradient Subcellular Fractionation Experiments

2.2.4.1 Homogenisation

A number of methods were tested to determine the optimum conditions for cell homogenisation of B16 murine melanoma cells. A brief list of the various parameters tested are listed as follows, and a more detailed procedure outlined below.

A. Choice of Homogenisation Method: 1) Tight-fitting dounce homogeniser
2) Loose-fitting dounce homogeniser
3) Needle and syringe

B. Choice of Homogenisation Buffer: 1) 1mM $NaHCO_3$; 1mM DTT
2) 1mM $NaHCO_3$; 1mM DTT; 0.25M sucrose
3) 0.05M Tris-HCl, pH 7.2
4) 0.05M Tris-HCl, pH 7.2; 0.25M sucrose

Note all homogenisation buffers contained 0.1mM PMSF and 1mM EDTA.

A Choice of homogenisation method

Tight-fitting Dounce Homogeniser

A confluent layer of B16 murine melanoma cells were scraped from a 75 cm^2 Falcon tissue culture flask using a cell scraper. These were pelleted by centrifuging at 1000 RPM for 10 minutes in a Jouan B3.11 Centrifuge. The cells were then suspended in PBS and

recentrifuged as before. This was repeated once more to ensure the cells were free from all the serum proteins present in the RPMI 1640 medium used for growth of the cells. The cells were then suspended in 0.75mls of the homogenisation buffer and left for 30 minutes at 4°C with regular shaking. The cell suspension was transferred to the tight-fitting dounce homogeniser and homogenised using 5, 10, or 15 strokes of the dounce homogeniser. A small sample was assayed to determine the number of cells still viable. The table below indicates the degree of cell lysis observed for 5, 10, or 15 strokes of the dounce homogeniser:-

NUMBER OF STROKES	VIABLE CELLS / ML	% LYSIS
0	1.59×10^6	0
5	0.71×10^6	55.5
10	0.29×10^6	81.6
15	0.01×10^6	100.0

Table 2.5: % Lysis of B16 Cells after Homogenisation using the Tight-Fitting Dounce Homogeniser.

Loose-Fitting Dounce Homogenisation

The same procedure as for "Tight-Fitting Dounce Homogeniser" was repeated, however a loose-fitting dounce homogeniser was used, and the number of strokes carried out were 0, 10, 20 and 30. The table below indicates the efficiency of this method:-

NUMBER OF STROKES	VIABLE CELLS / ML	% LYSIS
0	3.5×10^5	0
10	3.2×10^5	14.2
20	3.0×10^5	14.9
30	3.0×10^5	14.9

Table 2.6: % Lysis for B16 Cells after Homogenisation using the Loose-Fitting Dounce Homogeniser.

Needle and Syringe Method of Homogenisation

The B16 cells were scraped from the flasks, pelleted, washed and suspended in homogenisation buffer as for the "Tight-Fitting Dounce Homogeniser". The cell suspension was then passed through a series of needles of different aperature size. The viable cells were determined at various stages throughout this procedure to determine the % lysis obtained. Tables 2.7 & 2.8 overpage indicate the % lysis obtained after various treatments:-

METHOD 1:

TREATMENT OF CELLS	VIABLE CELLS / ML	% LYSIS
NONE	1.80×10^6	0
5 x 21G	0.77×10^6	43
+ 2 x 25G	0.15×10^6	92
+ 5 x 25G	0.01×10^6	100

Table 2.7: % Lysis of B16 Cells after homogenisation by Needle and Syringe by the Method specified.

METHOD 2:

TREATMENT OF CELLS	VIABLE CELLS / ML	% LYSIS
NONE	2.70×10^6	0
5 x 21G	1.66×10^6	39
+ 5 x 23G	1.24×10^6	55
+10 x 23G	0.16×10^6	94

Table 2.8: % Lysis of B16 Cells after homogenisation by Needle and Syringe by the Method specified.

For all three methods described, the homogenisation buffer used was 1mM $NaHCO_3$, 1mM DTT.

At this stage it was decided that the needle and syringe method for homogenising the cells would be used instead of a tight-fitting dounce homogeniser. Although both methods resulted in 82% lysis (tight-fitting dounce homogeniser using 10 strokes) and 94% lysis (needle and syringe method 2, table 2.8), both acceptable values, there were certain advantages to the needle and syringe method. Not only was it a fast and efficient method, it was also a much easier apparatus to dispose off. This was a major factor to consider due to the fact that there would be ^{125}I activity in the cells being homogenised after internalisation studies.

B Choice of Homogenisation Buffer

As stated at the beginning of this section four buffers were tested in an attempt to improve the efficiency of the process. The table below indicates the influence of each buffer after homogenising the cells by the needle and syringe method:-

CELL TREATMENT	BUFFER	% LYSIS
5×21G + 5×23G	1	94
"	2	69
"	3	72
"	4	84

Table 2.9: % lysis of B16 cells by various homogenisation buffers.

From these results it was decided to use the 0.05M Tris-HCl, pH 7.2; 0.25M sucrose (buffer D). Although little or no difference was apparent between buffer 1 and buffer 4, it was assumed that the hypotonic nature of the $NaHCO_3$/DTT buffer would result in more damage to the lysosomes. The addition of 0.25M sucrose to the Tris-HCl buffer not only helped its efficiency but also meant the sample was already suspended in a sucrose solution and so could be directly layered onto the gradient.

In conclusion to this section is a summary of the homogenisation method finally chosen:-

Needle and syringe : **5 × 21G + 5 × 23G**

Homogenisation buffer: **0.05M Tris-HCl, pH 7.2 / 0.25M sucrose**

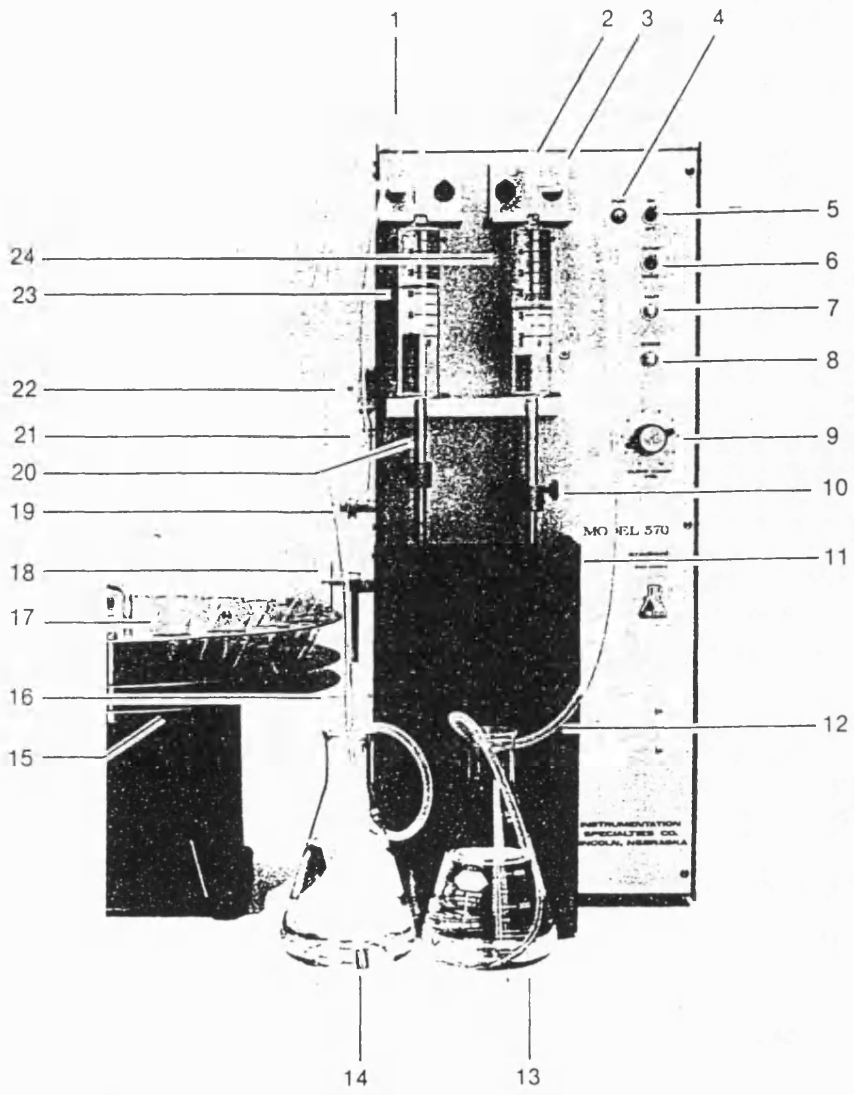
2.2.4.2 Centrifugation

Preparation of Linear Sucrose Gradient

A MSE Model 570 Gradient former was used to prepare the linear sucrose gradients ranging from density 1.000 to 1.300 gcm^{-1}. A photograph of the apparatus is shown on page 58.

To set the gradient former to the required state in order to obtain a 5ml linear gradient, the following protocol was followed:

Both syringes and all tubes were filled with double distilled water. The speed control was then set to maximum, i.e. 10. The right hand syringe delivery volume was set at 2.5mls and the gradient former switched on. Once the cycle was completed, the system was immediately switched off. The left hand syringe delivery volume was then set to 2.5mls also, and the machine switched on and the reverse button pressed. At the end of the cycle the machine was immediately switched off. The double distilled water was then flushed out of the system and replaced with the appropriate sucrose solutions. These were 60% in the right hand syringe and 10% in the left hand syringe. It was always necessary to ensure no air bubbles were in the system. To obtain a 5ml gradient ranging from 1.000 to 1.300 gcm^{-3}, the speed was adjusted to 3.5 and the machine switched on. Each gradient took 40 minutes to prepare and was collected in a 5ml centrifugation tube.



1	Make-Up Solution Delivery Tube	13	Reservoirs for Make-Up Solution (2)
2	Two-Way Valve(2)	14	Sinkers (2)
3	Top Syringe Clamp (2)	15	Fraction Collector Turntable
4	Pilot Light	16	Auto-Stop Switch
5	ON-OFF Power Switch	17	Centrifuge Tube
6	Single-Repeat Switch	18	Counter-Balanced Delivery Tube
7	Start Switch	19	Delivery Tube Shaker
8	Reverse Switch	20	Syringe Plunger (2)
9	Speed Control	21	Mixer
10	Syringe Plunger Clamps (2)	22	Air Purge Pump
11	Yoke Cover	23	Left Syringe
12	Valve to Reservoir Tubing (2)	24	Right Syringe

Figure 2.4: MSE Model 570 Gradient Former (Front View).

Centrifugation of Cell Homogenate

Approximately 0.5mls of the cell homogenate prepared in 0.05M Tris-HCl, pH 7.2 containing 0.25M sucrose was layered carefully on top of the linear gradient. This was best done using a pasteur pipette and gently running the cell homogenate down the side of the tube onto the top of the gradient. Each sample was loaded and carefully monitored to ensure the tubes would be balanced for the centrifugation stage.

Once layered onto the gradient, the linear gradients containing the homogenised sample were transferred to the SW40.1 rotor and centrifuged in a Beckmann Ultracentrifuge at 35,000 RPM for 90 minutes at 4°C.

2.2.4.3 Fractionation of the Linear Gradients

On completion of the 90 minute centrifugation, the tubes were carefully removed from the arms of the SW40.1 rotor. Special care was taken to avoid mixing of the gradients. Fractions of 200µl were then removed from the top of the gradient and transferred to LP4 gamma-counter tubes.

2.2.4.4 ^{125}I Activity of the Fractionated Cell Homogenate

The 200µl fractions obtained as described above were assayed for the counts per minute ^{125}I activity using a LKB 1277 Wallac Gammamaster automatic gamma-counter. The tubes were placed in the gamma-counter and the counts per minute determined for 120 seconds.

2.2.4.5 Density of the Centrifuged Fractions

The density of each fraction was determined by the indirect method of refractive index. A table of the refractive indexes of a number of different concentrations of sucrose are presented in Appendix A .

A drop of each fraction was placed on a prism and a light "step" aligned manually with cross-hair. The apparatus used for measurement of the refractive indices was maintained at 25°C to eliminate the effect change of temperature on the values obtained. The refractive indices were then converted into density from the table in Appendix A .

2.2.4.6 Enzyme markers

β-Hexosaminidase

50µl of the fraction was transferred to a 5ml test-tube and 100µl of the buffered substrate added. The volume was adjusted to 200µl with saline. After 4 hours at 37°C, the reaction was stopped by addition of 0.5ml of 50mM NaOH. The slightly turbid solution was clarified by centrifugation, and the absorbance measured at 400nm against a reagent blank using a Milton Roy Spectronic 601 Spectrophotometer.

Alkaline Phosphodiesterase I

The reaction mixture was prepared by mixing 0.1ml substrate solution, 0.1ml Tris-HCl buffer and 0.2ml water. 50µl of fraction was then added and the whole mixture incubated at 37°C for 2 hours. The reactions were then stopped by addition of 1.0ml glycine /

Na_2CO_3 , and their absorbance read at 410nm against a reagent blank using a Milton Roy Spectronic 601 Spectrophotometer..

2.2.4.7 Cell Binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH for Subcellular Fractionation Experiments

Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

B16 cells were grown to confluency on a 75cm^2 Falcon tissue culture flask. The cells were washed with ice-cold serum free RPMI 1640 medium prior to incubation with 0.1nM ^{125}I-[Nle^4,D-Phe^7]α-MSH at 37°C or 4°C for varying amounts of time, in binding buffer in the presence or absence of 1000-fold excess non-iodinated ligand. At the end of the required time, the binding buffer was removed and the cells thoroughly washed with ice-cold serum free medium. The cells were then scraped from the flasks and homogenised, centrifuged, fractionated and assayed for enzyme markers, density and ^{125}I activity. In a parallel flask a 5 minute acid treatment was carried out to distinguish between internalised ligand and externally bound ligand.

Pulse Chase Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

The same method was used as for cell well experiments (section 2.2.3.3), however cells were grown to confluency in a 75cm^2 flask and the binding carrried out on a confluent monolayer of cells. Homogenisation, centrifugation, fractionation, enzyme marker assays, density measurements and ^{125}I activity were assayed as described in the above sections.

CHAPTER 3

INTERNALISATION OF [^{125}I-TYR^{2}-NLE^{4},D-PHE^{7}]α-MSH.

3.1 INTRODUCTION

[Nle^{4},D-Phe^{7}]α-MSH is a potent melanotropin which stimulates tyrosinase, resulting in melanogenesis, after interacting specifically with a MSH (melanocyte-stimulating hormone) receptor at the plasma membrane of B16 murine melanoma cells. Previous workers in the field (Eberle, A.N.;1977) confirmed that [Nle^{4},D-Phe^{7}]α-MSH binds specifically to the MSH receptor and is 10-fold more potent than α-MSH itself. Although it has been reported that internalisation of the ligand after its initial binding occurs (Panasci, L.C. *et al*; 1987), no information exists on what happens after internalisation. The experiments described in this section, were designed to study the phenomenon of internalisation of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH (the mono-iodinated form), and once internalised, the fate of the ligand.

Internalisation was determined quantitatively by introduction of an acid dissociation technique. The theory behind this method is that the acid wash removes all ligand bound to the surface of the cell, including that bound to the plasma membrane. It would then be correct to say that any iodinated activity associated with the cells following the acid wash, was that present inside the cells, either in a degraded or non-degraded form. After internalisation was found to occur, it was then of interest to determine what happened to the ligand. The use of an exogenous amine, ammonium chloride was chosen to aid this field of study. Ammonium chloride, is known to trigger a pH rise in various acidic compartments of the cell, such as endosomes and lysosomes. The immediate consequence within the lysosomes is thought to be an

inactivation of proteolytic enzymes which have acidic optimum activity in media. Another consequence is thought to be general perturbation of vesicular traffic within the cells and of intracellular compartmentalisation resulting in modifications of the pathways of internalised materials, such as, hormone-receptor complexes. Therefore, if upon internalisation the [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH made its way to the lysosome and was subsequently degraded, the presence of NH_4Cl would inhibit such a pathway. Thus the presence of NH_4Cl (20mM) was introduced into the experiment in order to study the fate of internalised ligand. It must also be noted at this point, that the chapter following this deals with the fate of the internalised ligand by use of subcellular fractionation techniques.

One final aspect to be studied concerning the internalisation of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH dealt with was the incorporation of some protease inhibitors, namely leupeptin and pepstatin A. These would inhibit to some extent the enzymes responsible for the degradation of the ligand-receptor complex.

3.2 METHODS

These were described in detail in chapter 2.

3.3 RESULTS

3.3.1 Cell Binding of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH to B16 Melanoma Cells at 4°C

[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH (0.1nM), was bound to 5×10^5 B16 melanoma cells in a 24-well tissue culture plate for various periods of time at 4°C, and in the presence or absence of 10^{-6}M non-iodinated ligand. At the end of the required time,

the binding buffer containing unbound ligand was discarded and the layer of cells washed with ice-cold serum free RPMI 1640 medium. They were then removed by 1M NaOH digestion and the ^{125}I associated with the cells determined. A parallel series of wells was given the acid wash treatment, incubated for 5 minutes with 0.1M citrate buffer, pH 2.5 prior to digestion with NaOH.

Figure 3.1 on page 69 represents the data obtained for binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 4°C. All data points represent the mean and standard deviation from four wells and show a very small degree of variation. Non-specific binding was assessed in the presence of 10^{-6}M non-iodinated [Nle^4,D-Phe^7]α-MSH. Specific binding was obtained by subtracting the non-specific binding from the total binding.

At 4°C specific binding was still increasing at 4 hours, as was non-specific binding. The amount of ligand associated with the inside of the cells remained low compared with specific binding and this would suggest that at 4°C, little or no internalisation occurred.

3.3.2 Cell Binding Of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to B16 Melanoma Cells at 37°C

The experiment to study the cell binding of the ligand at 4°C (as described in section 3.3.1) was repeated at 37°C. The results are shown in Figure 3.2 on page 70.

The data obtained at 37°C was noticeably different to the 4°C results. At 37°C, the apparent specific binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH reached a maximum at 60 minutes and then decreased for the remaining time of the experiment. The acid-wash data (internalised ligand) showed a similar result to the specific binding. An increase in activity was evident between 0 and 60 minutes, and a gradual decrease occurred after this time. It must be noted that the amount of ligand within the cell decreased to a

lesser degree than that specifically bound. In fact at 240 minutes more than 70% of the ^{125}I activity associated with the cells at the 60 minute time period was present in the case of the acid treated cells. This compares to only 50 % for the same time period differences for the ligand specifically bound to the cells. At 37°C, [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was internalised by the B16 cells, however, it has not been determined whether this is in a complete or fragmented form. After 60 minutes, 40% of the cell-associated activity was within the cells.

3.3.3 The Ammonium Chloride Effect on the Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with B16 Melanoma Cells

The experiment described in section 3.3.2 confirmed that internalisation of the ligand occurred at 37°C. It was then necessary to determine the fate of the ligand, and the reason for the decrease in the ligand associated with the cells after 60 minutes.

One reason for such a phenomenon occurring, may be that the ligand was degraded within the cells. Hence, the incorporation of NH_4Cl, a lysosomotropic agent into the system. NH_4Cl raises the pH of the lysosomes hence inactivating proteolytic enzymes with acidic pH optima.

A "binding" experiment was performed at 37°C, and 20mM NH_4Cl was introduced into the system. A pre-incubation of the cells at 37°C in serum free RPMI 1640 medium containing 20mM NH_4Cl was carried out, and all buffers thereafter contained 20mM NH_4Cl.

The graph in Figure 3.3 on page 71 is a typical experiment of the binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C in the presence of 20mM NH_4Cl. Non-specific binding remained low throughout the 240 minutes duration of the experiment. This

correlates with what was observed in the same experiment but in the absence of the NH_4Cl. However, the specific binding and acid-wash data differed greatly in the presence and absence of the NH_4Cl. Specific binding continued to increase over the whole 240 minute period, as did the acid-washed values. Moreover, at 240 minutes, 90% of the cell-associated ligand was internalised, compared with only 40% in the experiment not containing any NH_4Cl (Figure 3.2).

3.3.4 Time Dependence of the Internalisation of the [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 Melanoma Cells

Here a set of experiments were set up to study more closely the time course of the internalisation of the [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. They involved an incubation of the cells for 2 hours at 4°C in the presence of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. After this time, the unbound ligand was removed and the cells subjected to a second incubation in serum free RPMI 1640 medium at 37°C for various times and without any additional iodinated ligand. In this way the ligand was allowed to bind to the exterior of the cells (remembering that no internalisation occurred at 4°C as was confirmed in section 3.3.1) and then its internalisation monitored over a 90 minute period at 37°C.

The results of this experiment are shown in Figure 3.4, page 72. As for the previous experiments described in section 3.3.1 to 3.3.3, the data plotted represented the mean and standard deviation for four wells.

From Figure 3.4, specific binding gradually decreased throughout the entire 90 minute period of the 37°C incubation. 60% of the initial ligand bound to the cell-surface had disappeared and only 25% of the remaining 40% was internalised. The acid-wash values interestingly showed an initial increase from none or a negligible amount of

^{125}I-activity internalised into the B16 cells to a maximum at 30 minutes. By 90 minutes however a third of this (31%) had dissociated from the cells again. This could be due to degradation of the ligand or recycling - a question not answered yet. Non-specific binding as usual, remained relatively low throughout the entire 90 minutes.

To determine what was happening during the 37°C incubation the effect of NH_4Cl was again introduced into the experiment. The same experiment as that shown in Figure 3.4 was carried out, in the presence of 20mM NH_4Cl and the data is displayed in Figure 3.5 on page 73. Again non-specific binding was relatively low throughout the experiment. Specific binding was constant between 10 and 90 minutes though there was a loss of binding during the first 10 minutes. This initial decrease in binding was probably due to desorption. However, the acid-wash data differed to a great extent. The ^{125}I-activity internalised within the cells appeared to increase gradually and was still rising at 90 minutes. In fact at 90 minutes, 100% internalisation had occurred, shown by the fact that all the specifically bound ligand was now within the cells.

3.3.5 The Effect of Leupeptin on the Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH

Due to the results obtained from the effect of ammonium chloride on the internalisation of the ligand (which indicated that degradation could be occurring in the lysosomal compartment), leupeptin was introduced into the system. This is a protease inhibitor, and its effect would be to inhibit any trypsin-like enzymes in the lysosome from functioning properly.

A 10μgml^{-1} concentration of leupeptin was included in the experimental buffers. As for NH_4Cl, the cells were subjected to a 15 minute pre-incubation at 37°C with leupeptin in serum free RPMI 1640 medium. All buffers thereafter contained 10μgml^{-1} leupeptin.

Figure 3.6a on page 74 shows the data obtained for this experiment. There was no apparent difference in the internalisation of the ligand when compared to the same experiment carried out in the absence of leupeptin (Figure 3.6b, page 75). This may indicate that trypsin-like enzymes are not responsible for the breakdown of the internalised material.

3.3.6 The Pepstatin A Effect on the Internalisation of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH

Pepstatin A is another protease inhibitor introduced to study its effect on the internalisation of the ligand. Pepstatin A has a wider spectrum of use, but inhibits aspartic proteases in particular. The same experiment was repeated as in section 3.3.5, however, 10μgml^{-1} pepstatin replaced the 10μgml^{-1} leupeptin.

The results of this experiment are shown in Figure 3.7a and 3.7b on pages 76 and 77 respectively. As with the leupeptin experiment in section 3.3.5, no apparent differences in the internalisation of the ligand was observed. This indicates that the enzymes inhibited by this pepstatin A are not those responsible for the degradation of any internalised material.

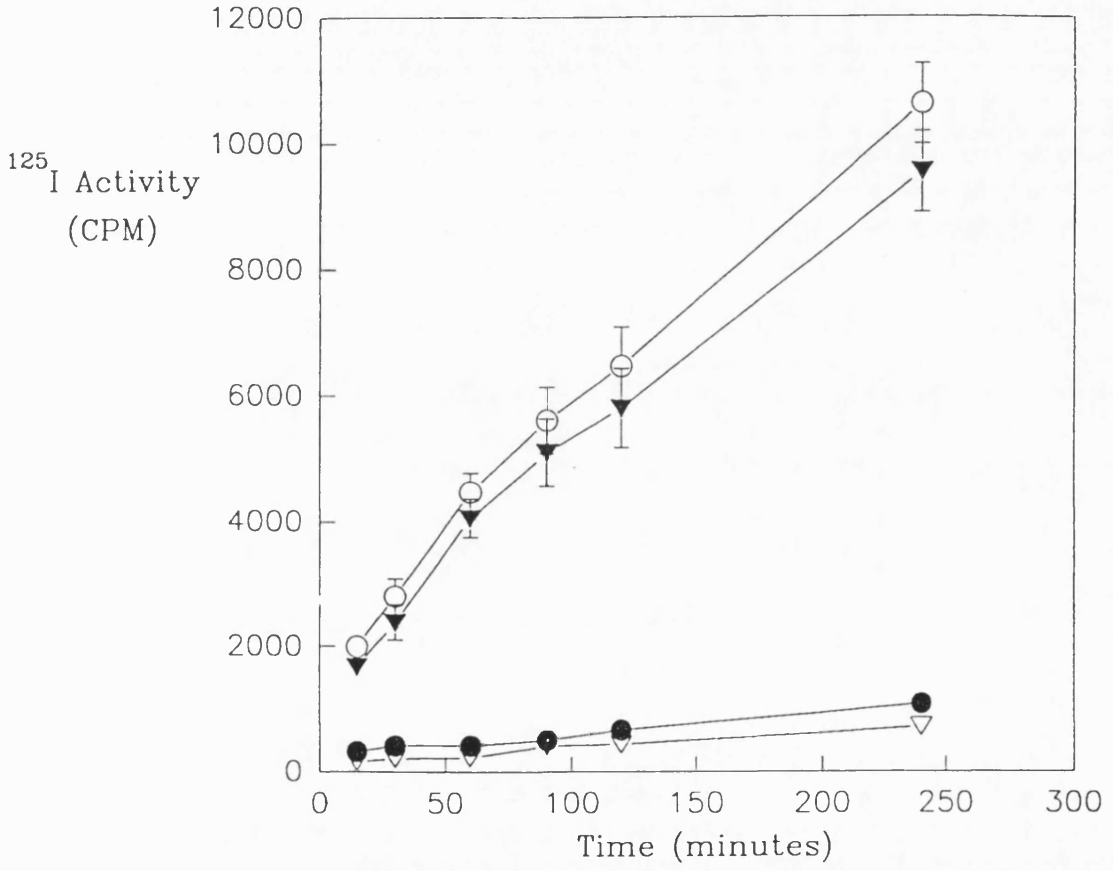


FIGURE 3.1: ^{125}I Activity after incubation of 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH with B16 melanoma cells at 4°C with time in a HEPES based buffer, pH 7.4. Each point represents the mean ± s.d. from 4 wells. Total (○), specific (▼), non-specific (▽) binding respectively and internalised ligand (●).

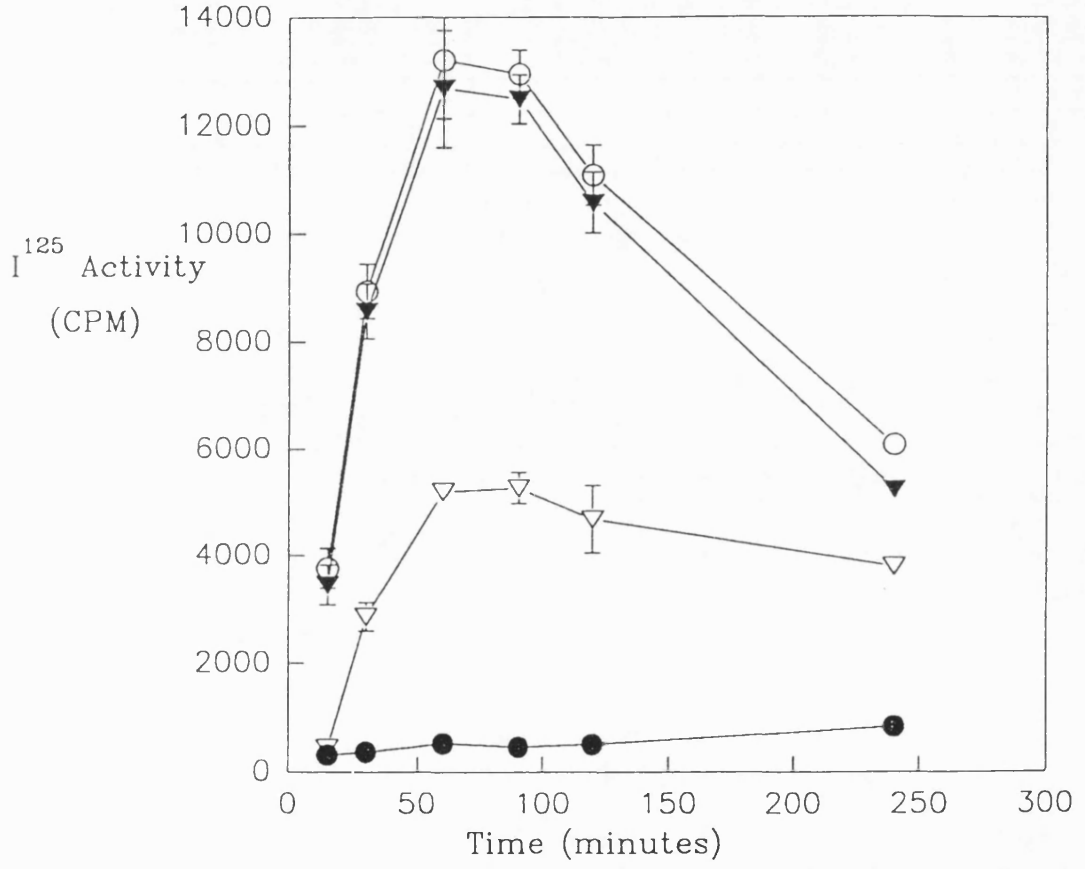


FIGURE 3.2: ^{125}I Activity after incubation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with B16 melanoma cells at 37°C with time in a HEPES based buffer, pH 7.4. Each point represents the mean ± s.d. from 4 wells. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

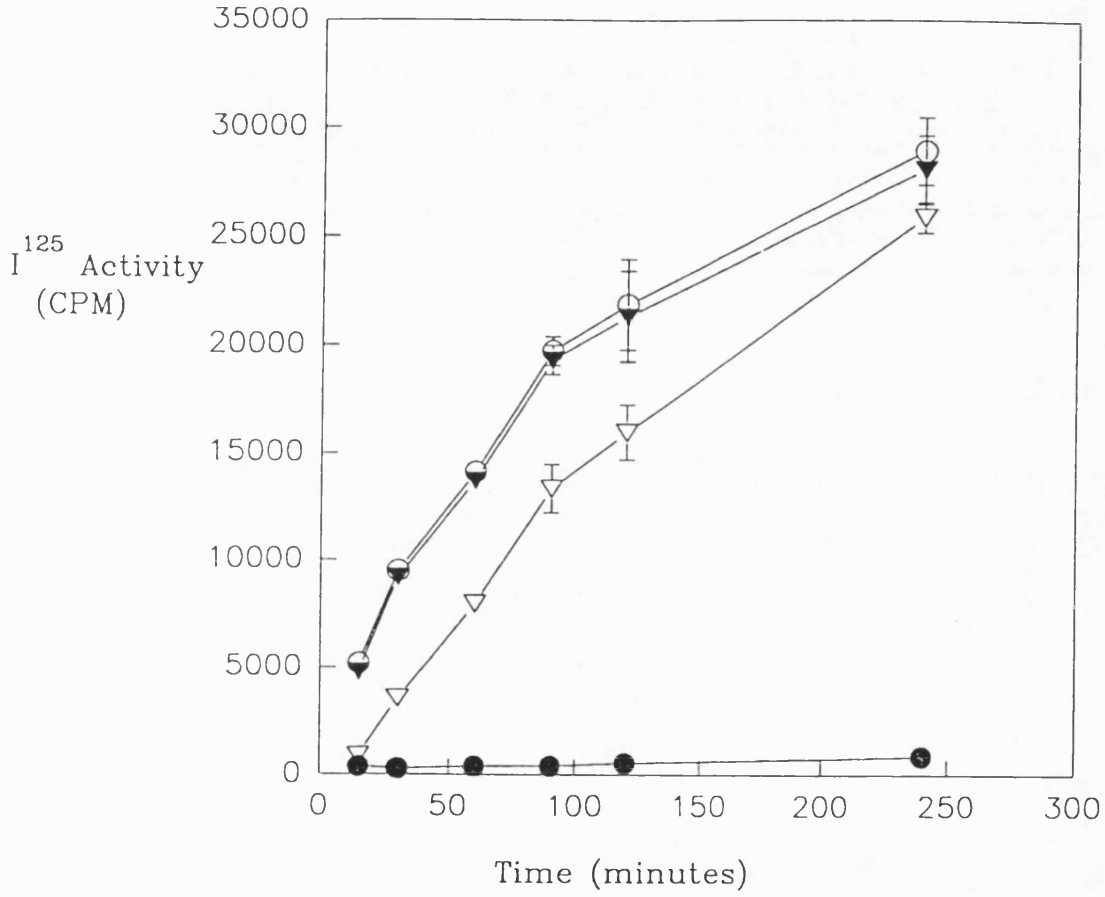


FIGURE 3.3: ^{125}I Activity after incubation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with B16 melanoma cells at 37°C with time in a HEPES based buffer, pH 7.4 in the presence of 20mM NH_4Cl. Each point represents the mean ± s.d. from 4 wells. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

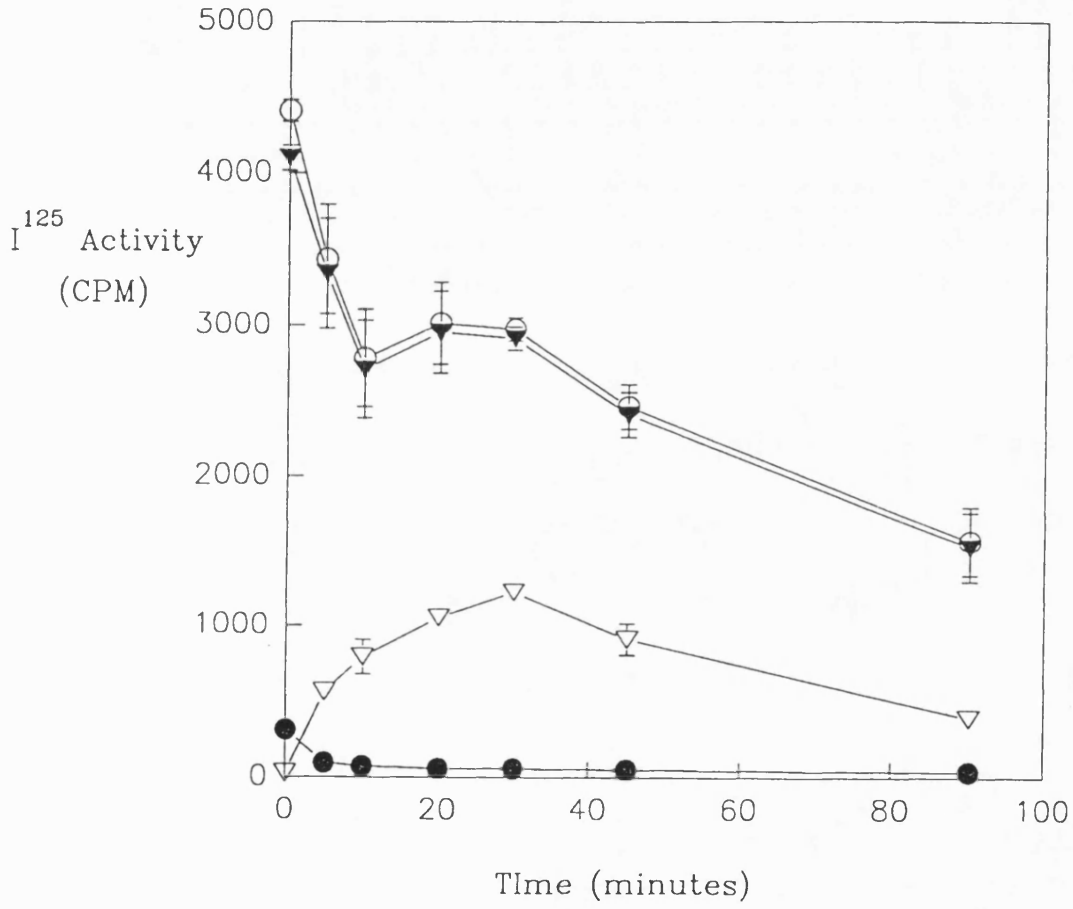


FIGURE 3.4: Time dependence on the internalisation of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH into B16 melanoma cells after binding of 0.1nM of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

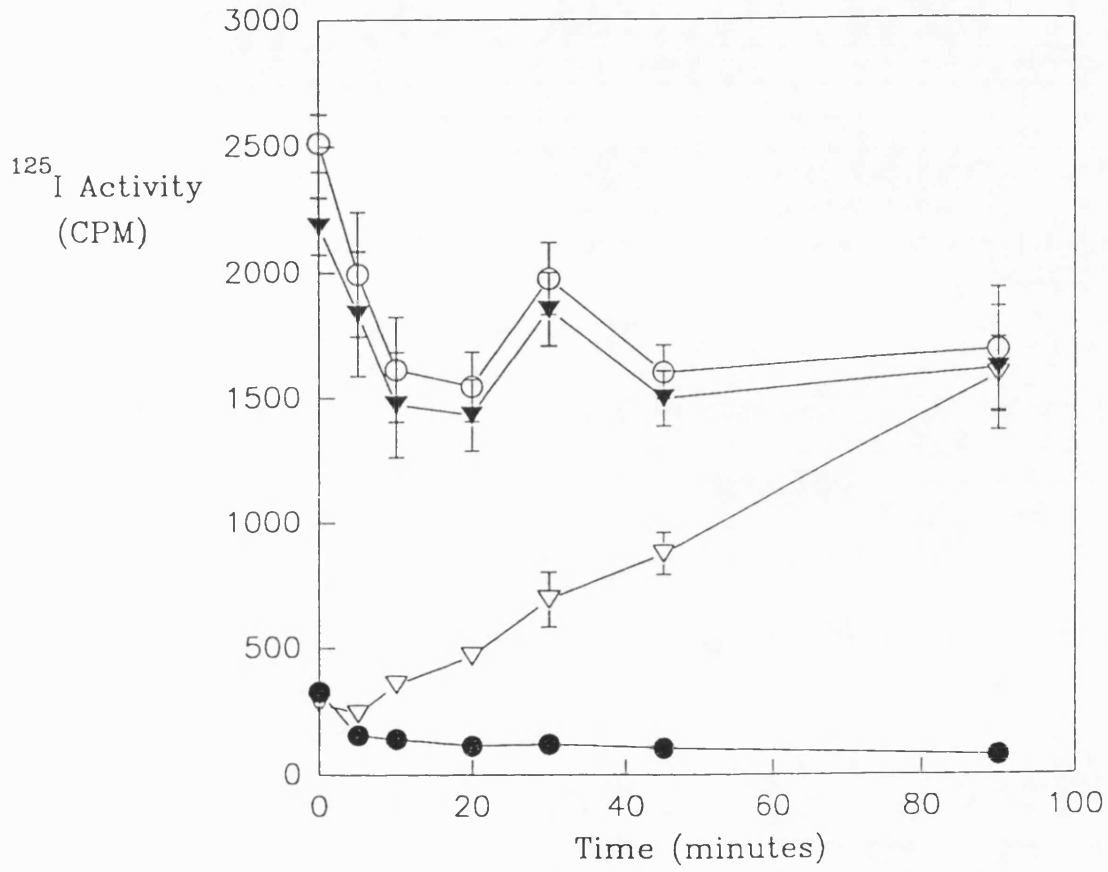


FIGURE 3.5: Time dependence on the internalisation of ^{125}I-[Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM of ^{125}I-[Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the presence of 20mM NH_4Cl. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

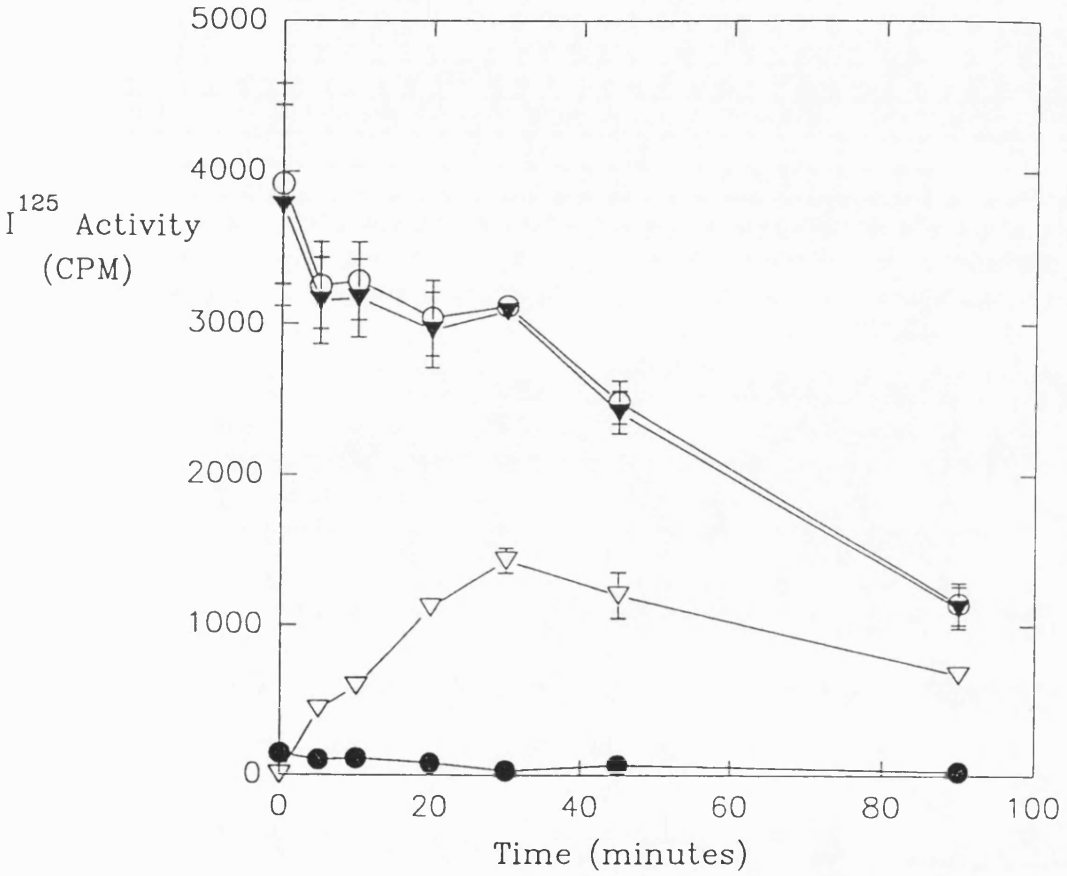


FIGURE 3.6a: Time dependence on the internalisation of ^{125}I-[Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM of ^{125}I-[Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the presence of 10μgml^{-1} leupeptin. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

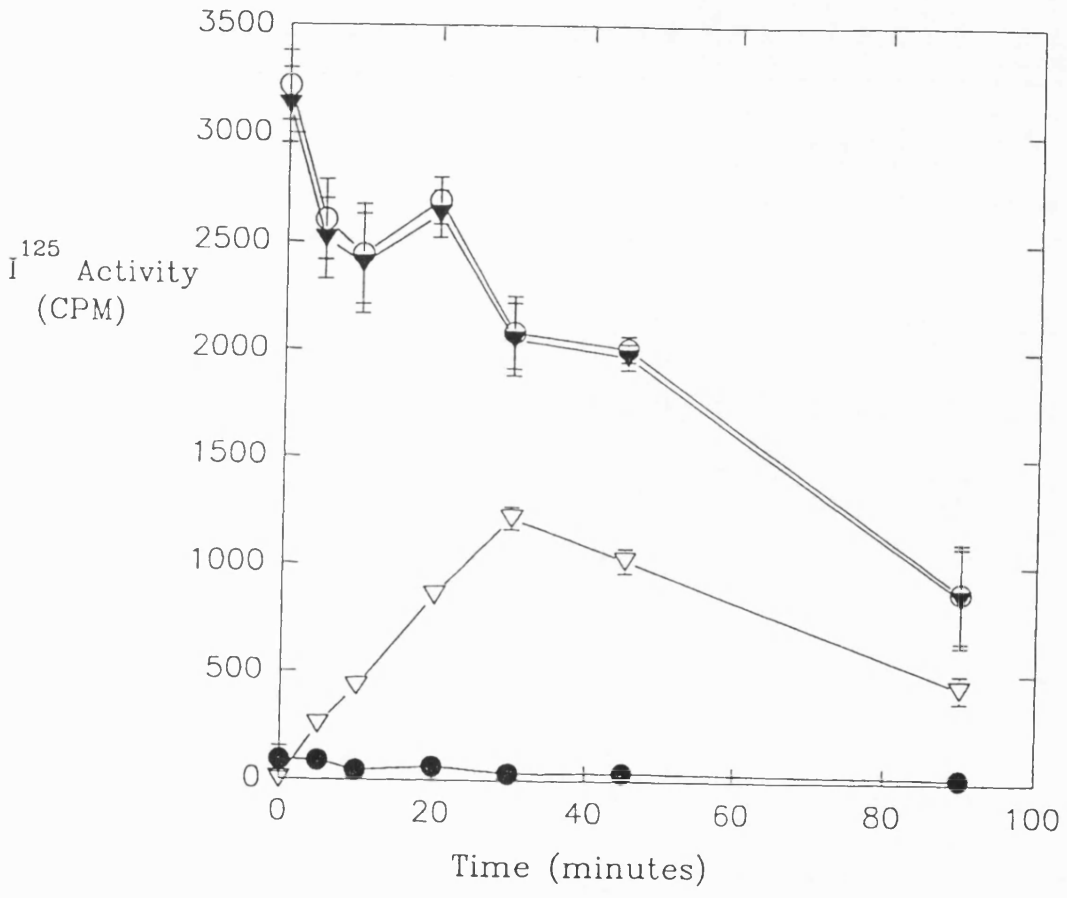


FIGURE 3.6b: Time dependence on the internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the absence of 10μgml^{-1} leupeptin. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

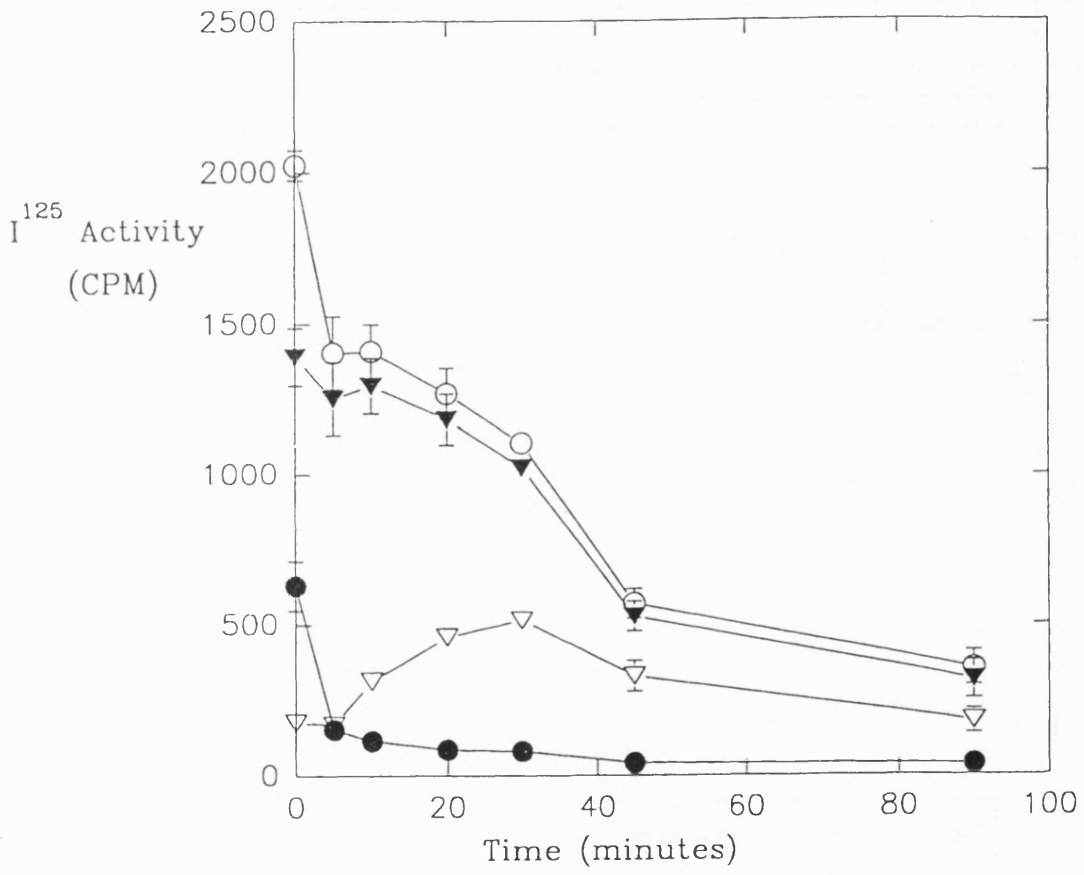


FIGURE 3.7a: Time dependence on the internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the presence of 10μgml^{-1} pepstatin A. Total (○), specific (▼), non-specific (▽) binding respectively and internalised ligand (●).

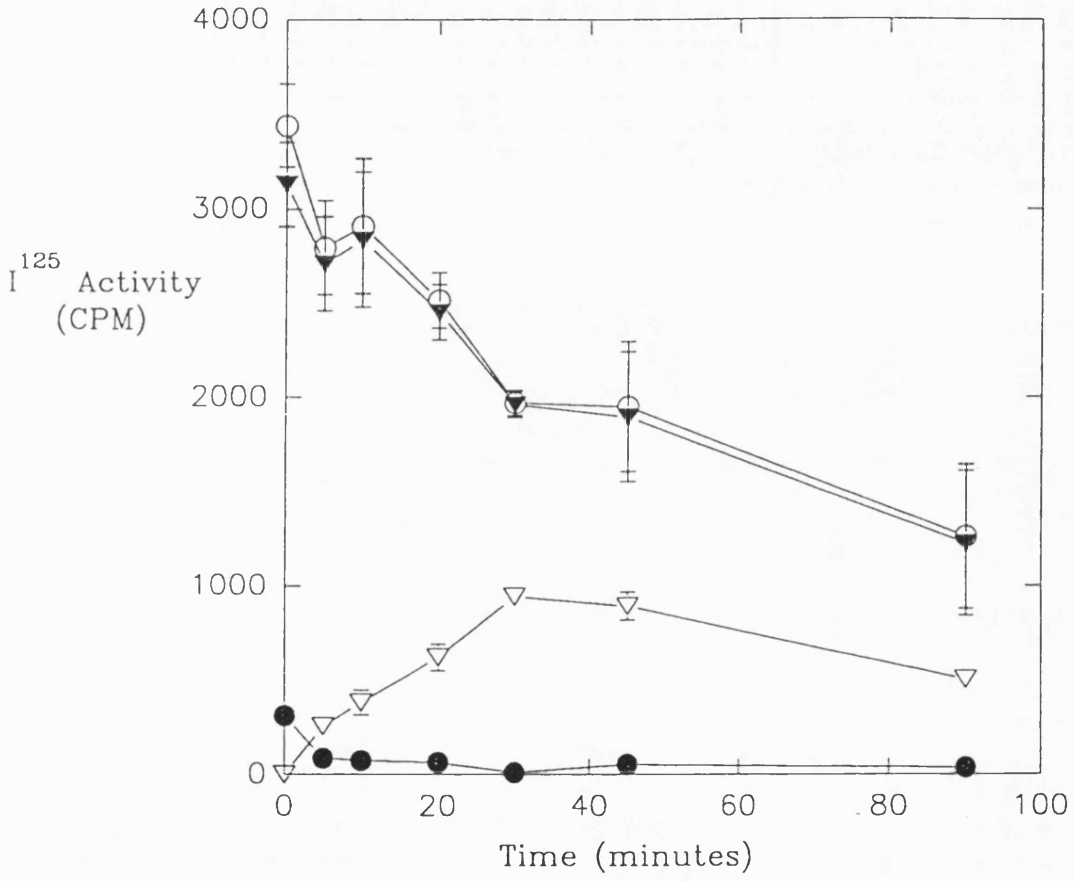


FIGURE 3.7b: Time dependence on the internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the absence of 10μgml^{-1} pepstatin A. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

3.4 DISCUSSION

[Nle^4,D-Phe^7]α-MSH has been shown by Sawyer *et al* (1982), using bioassays, to have superagonist potency and an extraordinary prolonged biological activity compared with the native hormone, α-MSH. It was resistant to degradative inactivation by serum enzymes (Sawyer, T.K. *et al;* 1980) and hence proved very useful for the studies regarding cellular binding and internalisation reported here in section 3.3. Previous workers in this area, had suggested binding occurred at the cell surface without internalisation (Eberle, A.N. *et al;* 1977, Legros, F. *et al;* 1981), however the results reported by Panasci *et al* (1987) challenged this. Panasci and his colleagues, although using ND_{4-11}α-MSH and a F1 variant cell-line, found binding and internalisation to occur. The results obtained here using [Nle^4,D-Phe^7]α-MSH and B16 cells are in agreement with these findings.

From the initial experiments performed, it was concluded that internalisation only occurred at 37°C and not at 4°C. At 4°C, cellular binding did occur, however, the acid treated cells showed a negligible amount of ^{125}I-activity, implying that no internalisation of the [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH had occurred. For the 37°C experiment, the specific binding and internalised ligand gradually increased from 0 to a maximum at 60 minutes. This was followed by a decrease for the remainder of the experiment. These results agree with the data of Panasci *et al* (1987) where they used an acid-wash to distinguish between extracellularly bound and intracellular associated ligand. They too reported a maximum amount of internal ligand at 60 minutes, followed by a decrease.

From these results questions arose: the prime question being what causes this decrease after 60 minutes? There could be a few reasons for such a pattern, the most likely of which was the involvement of degradation. The use of ammonium chloride which acts

as a lysosmotropic agent increasing the pH of the endosomes and lysosomes hence restricting lysosomal enzyme degradation and preventing vesicle fusion events in the endocytic pathway, (Muller, J-M. *et al;* 1985) was therefore introduced. A sharp difference in the acid treated cells was observed, between those subjected to NH_4Cl and not subjected to NH_4Cl. Instead of reaching a maximum ^{125}I-activity associated with the cells at 60 minutes, after NH_4Cl treatment the radioactivity present was still increasing even at 240 minutes, implying that the internalised ligand was still within the cells. The specific binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH also continued to increase throughout the duration of the experiment. This increase of internalised ligand in the presence of ammonium chloride could be accounted for by non-degradation resulting in an accumulation, or non-dissociation from internalised receptors trapped within the cells.

It should be noted that a second reason for this decrease in activity and apparent loss of internalised ligand could be the internalisation of the ligand-receptor complex which would ultimately result in a temporary loss of membrane binding sites. A technique whereby the receptor could be labelled covalently and then monitored after binding to the cell surface would help test this hypothesis.

Although the experiments carried out at 37°C confirmed that internalisation did occur for the system of B16 melanoma cells and [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH ligand, it was not possible to estimate the rate of internalisation with time. Hence, the second series of experiments were performed. These introduced the "pulse chase" idea, whereby, [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was initially bound to the B16 cells at 4°C, unbound ligand removed after 2 hours and the cells were then subjected to a 37°C incubation. If the ligand still appeared internally, it could then be almost certain that it entered the cells via the α-MSH receptor since it was bound to the cell surface receptor during the 4°C incubation in the presence of the iodinated ligand.

The data from such experiments did show the internalisation of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH. At 0 minutes, no ligand was apparent within the cells but a gradual increase occurred up to 30 minutes, after which a decline was observed. The total specific association on the other hand, decreased during the whole 90 minutes of the incubation at 37°C. These observations were most likely caused by internalisation of the surface bound ligand (from the 4°C incubation), and subsequent degradation of the ligand or recycling of the receptor-ligand complex.

To probe the phenomenon of degradation, the use of ammonium chloride was again introduced. Interestingly, the acid treated cells showed a continuous increase in ligand associated with them over the entire 90 minute period of the experiment. The specific-binding, was constant between 10 and 90 minutes. Approximately 100% of the ^{125}I activity associated with the B16 melanoma cells after 90 minutes appeared to be internal. These results further establish the theory that the decreases in cell associated activity in the absence of NH_4Cl were due to degradation by lysosomal enzymes. So overall, the results obtained in the presence of ammonium chloride indicate that the decrease in internalised ligand was pH mediated and that it occurred in a relatively acidic endocytic environment, such as the lysosome.

As it was considered possible that the decrease in cell-associated activity of the [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH with the B16 melanoma cells, could be caused by degradation occurring in the lysosomal compartment, it was decided to study the system with protease inhibitors present. The two inhibitors chosen were leupeptin and pepstatin A. Leupeptin inhibits cysteine / serine proteases and is effective at micromolar concentrations. Pepstatin A inhibits aspartic proteases and is again effective at micromolar concentrations (North, M.J.; 1989). When these were present in the experimental set-up, no noticeable difference was observed. Although this may imply that the proteases inhibited do not cause the degradation, further work should be

carried out in this area. In practice there is considerable variation in the concentrations of the inhibitors used by different workers and hence, the concentration used in the experiments studied here may not be suitable. However, due to the high cost of these inhibitors, $10\mu gml^{-1}$ was the chosen concentration. Harford & Klausner (1987), when working with hepatocytes to study receptor-mediated endocytosis, using a concentration of $100\mu gml^{-1}$ leupeptin, found radioactivity accumulating in lysosomal regions of a subcellular fractionation gradient.

Unfortunately there was no available time to further this area of study. In parallel to this work however, subcellular fractionation studies were set-up. This was in order to achieve a greater insight into the internalisation process and internalisation. The work carried out on this area is cited in Chapter 4.

In conclusion, it can be said that [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH was internalised in a temperature-dependent manner.

CHAPTER 4

FRACTIONATION OF B16 MELANOMA CELLS AND THE FATE OF [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH.

4.1 INTRODUCTION

[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH was internalised after binding to its cell-surface receptor was concluded in Chapter 3 from an extensive study involving cell binding experiments carried out in well plates. It was determined that after 30 minutes the maximum amount of ligand had been internalised, and by 90 minutes the amount of ligand within the cell was greatly reduced. From experiments carried out in the presence of ammonium chloride (an exogenous amine known to trigger a pH increase in various acidic compartments including the lysosome), it was observed that the ligand was still within the cells even as long as 90 minutes, and indeed was still increasing at this time. These results tend to suggest that the ligand is internalised rapidly and is degraded within 30 to 90 minutes after internalisation.

However, it is necessary to study where the ligand travels to once internalised to be able to conclude that this is what is actually happening. In order to do this, subcellular fractionation experiments were set up. These involved the introduction of density gradient centrifugation techniques, where a combination of rate zonal and isopycnic techniques were used. Density gradient was chosen over normal differential centrifugation due to its permission of analytical measurements to be made, in addition to complete separation of several or all of the components in a mixture. The density gradient method involves a

supporting column of fluid whose density increases towards the bottom of the tube. The density gradient fluid consists of a suitable low molecular weight solute in a solvent in which the sample particles can be suspended. In the experiments described below, a combination of rate zonal and isopycnic techniques were incorporated whereby the gradient was of such a density range that one component sedimented to the bottom of the tube, while another component sediments to its isopycnic position and remains there.

Density gradients are used extensively in the separation and purification of a wide variety of biological materials. Cells, and cell-particulate fractions including erythrocytes, spermatozoa, lymphocytes, bacteria, spleen cells, nerve cells, and the nuclear, mitochondrial, microsomal, and supernatant fractions of mammalian tissues have been isolated and purified (Birnie, G.D.; 1972; deDuve, C.; 1971). The enzymatic activities associated with these fractions, as well as with the less discrete lysosomal and peroxisomal fractions, have been measured after density gradient centrifugations.

As was discussed in Chapter 1 of this thesis, sucrose was chosen as the gradient material. Table 4.1 overpage gives a list of appropriate densities of some biological particles in sucrose solution.

MACROMOLECULES	DENSITY (gcm^{-3})
GOLGI APPARATUS	1.06 - 1.10
PLASMA MEMBRANES	1.16
SMOOTH ENDOPLASMIC RETICULUM	1.16
MITOCHONDRIA	1.19
LYSOSOMES	1.21
PEROXISOMES	1.23

Table 4.1: Approximate Densities of Macromolecules in Sucrose Solutions.

In theory, the values of density of the plasma membrane ($1.16gcm^{-3}$) and that of the lysosomes ($1.21gcm^{-3}$) were considered to be different enough to get a clean separation after centrifugation. If this were so, then it would then be possible to determine if the ^{125}I-ligand was simply bound to the cell surface (and would therefore be present in the plasma membrane fraction) or was within the cells (and migrated to the lysosomal fraction). In order to confirm the exact density of these organelles for B16 melanoma cells, enzymes assays were set up. For plasma membrane alkaline phosphodiesterse I was chosen to determine its position in the sucraose gradient. To locate the lysosomes, β-hexosaminidase was used. Details of experimental procedures for these marker enzymes are discussed in Chapter 2, section 2.2.4.6, on page 59.

The experiments designed in this case were such as to try and determine the position of the ligand once it had undergone internalisation, and if possible calculate the quantity of it internalised. As was described in Chapter 2, section 2.2.4.1, extensive work was performed on the homogenisation of the B16 melanoma cells in an attempt to optimise the

disruption of the cells. Severe enough to give adequate separation of the relevant organelles, but gentle enough so as not to damage any of them. In addition to this, once the separation had been carried out by density gradient centrifugation, the fractions associated with plasma membrane or lysosome had to be defined. This was done by enzyme assays. Refractive indices were also determined as an indirect measurement of the density of each fraction.

Eventually a procedure for subcellular fractionation of B16 murine melanoma cells was established. The optimum homogenisation with the equipment available; and fractions containing plasma membrane and lysosome were defined.

4.2 METHODS

These are described in detail in Chapter 2.

4.3 RESULTS

4.3.1 Linearity of Sucrose Gradients

The first step in this series of experiments was to establish a method for forming a linear sucrose density gradient in a repetitive manner. To do this a number of gradients were prepared as described in section 2.2.4.3. These were then subjected to centrifugation, 35000 RPM at 4°C for 90 minutes, and then fractionated into 200µl fractions. The refractive index of these fractions were carried out, and subsequently the density obtained from the table displayed in Appendix A.

A representative graph of density against fraction number is displayed in Figure 4.1 on page 93. This clearly shows that a linear gradient between 1.04gcm^{-3} and 1.20gcm^{-3} can be produced .

This procedure was repeated in the presence of homogenised B16 melanoma cells. Again linear gradients were observed , thus confirming that a suitable gradient and centrifugation had been established for the fractionation of these cells.

4.3.2 Enzyme Assays

Once a suitable method for forming the gradients had been achieved, it was then essential to define the position of the relevant organelles, namely, the plasma membrane and the lysosome for the B16 melanoma cells. In order to do this enzyme assays were introduced.

There are certain enzymes characteristic of each subcellular organelle and by assaying each fraction of the gradient for a particular one associated with a certain organelle, it could be determined at which density the plasma membrane and lysosomes were being separated out into. The initial enzymes studied for this purpose were β-glucuronidase (lysosomal marker) and 5'-nucleotidase (plasma membrane marker). Unfortunately neither of these give the required sensitivity for the fraction size under analysis. Therefore it was necessary to look at other marker enzymes which may provide the sensitivity necessary for this system. In referance to Muller, J.M. *et al* (1985), β-hexosaminidase and alkaline phosphodiesterase I were next chosen. These were representative of the lysosome and plasma membrane respectively. Details of experimental protocols for these assays are described in section 2.2.4.6, on page 59.

The graphs on pages 94 and 95 (Figure 4.2a and 4.2b respectively), represent the enzyme marker profiles along the course of the gradient. It must be noted that relative absorbance units are stated as a measure of the amount of enzyme within each fraction assayed.

From Figures 4.2a and 4.2b on pages 94 and 95 respectively, it can be concluded that a satisfactory method for the enzyme markers assays for the plasma membrane and lysosome of B16 melanoma cells had been achieved. It was observed that the plasma membrane marker appeared to be predominant in fractions 1 to 6, however, this was most likely due to soluble enzyme either from the cytoplasm or detached during homogenisation. A second area of plasma membrane marker occurrence, was between fractions 18 to 22 (density range 1.15 to 1.18gcm^{-3}). For β-hexosaminidase, the major areas of interest appear in fractions 14 to 20 (densities 1.16 to 1.18gcm^{-3}); the expected density range for lysosomes.

4.3.3 Internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH and its Fate once within the Cells Monitored by Subcellular Fractionation Studies

In order to determine if B16 melanoma cells could successfully be fractionated and the ^{125}I activity associated with each fraction measured, the following experiment was performed.

B16 melanoma cells were grown to confluency on 75cm^2 Falcon tissue culture flasks. The cells were subjected to a 2 hour incubation at 4°C or a 30 minute incubation at 37°C in 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. This was carried out in the presence and absence of 1000-fold excess non-iodinated ligand, and parallel flasks were set up to determine the extent of internalisation (by the acid washing technique previously used for the cell well experiments). At the end of the incubation, the unbound ligand was discarded and the cells thoroughly washed with ice-cold serum free RPMI 1640 medium. The cells were then scraped from the flasks and homogenised, centrifuged and fractionated as described in sections 2.2.4.1 and 2.2.4.2.

The graphs displayed in Figures 4.3 and 4.4 represent the fractionation of B16 cells subjected to a 4°C and 37°C incubation respectively. The 4°C data (Figure 4.3, page 96) relates favourably with that obtained with the cell well experiments in that no internalisation has apparently occurred. The activity associated with the fractionated cells appears in the initial fractions of the sucrose gradient, those where the plasma membrane predominates. Note there is a small peak at fraction 20, possibly due to trapping of the plasma membrane, or too severe a homogenisation. Little or no ^{125}I activity is observed internally in later and more dense fractions associated with intracellular organelles. In contrast Figure 4.4, on page 97, the repeat experiment at 37°C shows a different profile of

radioactivity associated with each fraction. In this case, the ^{125}I appears to be more pronounced in a more dense region of the gradient (fractions 16 and above). In comparison with figure 4.3, the acid-resistant ^{125}I activity is more defined with a peak around fractions 16 to 21, those known to be associated with internal organelles, lysosomes included. No such peak of activity was observed for the 4°C experiment.

The results obtained from this experiment would tend to suggest that separation of the organelles has been achieved to a degree high enough to allow observation of internalised ligand and that associated with the exterior of the cells. The acid treated cells display no internalisation at 4°C, that is, no ^{125}I activity associated with the lysosomes and at 37°C the opposite occurs.

4.3.4 Time Course Experiment of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to Follow the Fate of the Internalised Ligand Using Sucrose Density Gradient Subcellular Fractionation

From the results obtained in section 4.3.3 above, separation of internalised and surface-bound [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was successfully accomplished by subcellular fractionation. A series of experiments were then designed to follow the path of the ligand after binding at 4°C. These were similar to the "pulse chase" experiments described in section 3.3.4. Cells were grown to confluency on 75cm^2 Falcon tissue flasks and incubated for 2 hours at 4°C in 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. After washing with serum free RPMI 1640 medium, they were subjected to a second incubation at 37°C in serum free RPMI 1640 media in the absence of any iodinated material. At the end of the required time at 37°C the cells were scraped from the flasks and fractionated as described earlier (section 2.2.4.1 to 2.2.4.3).

The graphs displaying the data obtained for this study are shown on pages 98 to 101. Figure 4.5a represents the profile of radioactivity observed for no incubation at 37°C, that is to say it shows what happened after the 4°C incubation. Although a high peak of ^{125}I activity was observed at the beginning of the gradient this was not thought to represent intact plasma membrane. It was the peak around 16 to 22 which was of greater interest, which represented the denser frigement. When an acid wash had been carried out no such peak was observed suggesting any [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH bound to the melanoma cells was surface bound and could be removed by the acid wash treatment.For the next two timepoints, the 15 minute and 30 minute incubations at 37°C, figures 4.5b

and 4.5c respectively, the major observation was the failure of the acid wash to remove the peak of ^{125}I activity in the denser fractions. This would indicate that at these times the ligand had moved from the outer surface of the cell inwards. Interestingly, the 60 minute timepoint (figure 4.5d) showed a highly reduced amount of ^{125}I activity, and no defined areas of increased activity. This would imply that by 60 minutes at 37°C the ligand had either left the cells or had been degraded.

To confirm if degradation was occurring, the use of NH_4Cl was again introduced as had been in the cell well experiments. The same experiment as above was repeated, but in the presence of 20mM NH_4Cl. The results are displayed in Figure 4.6a-d on pages 102 to 105.

As in the experiment shown in Figure 4.5a-4.5d, the graphs labelled a, b, c and d are for 0, 15, 30 and 60 minutes incubation at 371°C respectively. As in figure 4.5a, the experiment carried out in the absence of NH_4Cl, figure4.6a representing the same experiment but in the presence of NH_4C all peaks of ^{125}I activity shown to be associated with the cells are removed in the presence of an acid wash indicating once again that at 4°C, all the ^{125}I associated with the B16 melanoma cells was extracellularly bound. For the 15 minute and 30 minute timepoints (figures 4.6b and 4.6c respectively) very similar results were obtained as for the equivalent timepoints in the absence of the NH_4Cl (figures 4.5b and 4.5c). Not all the ^{125}I associated with the cells was removed by an acid wash again , indicating the transfer of surface bound ligand to the inside of the cells. The 60 minute timepoint (figure 4.6d) showed clearly the effect of NH4Cl. Unlike figure 4.5d where a wide spread of low 125I activity was observed, there was an abundant peak of 125I activity associated with the cells, not all of which was removed by an acid wash. This

would indeed further suggest that the lysosome was in some way connected with the decrease in cell associated activity at the higher timepoints, most likely, degradation.

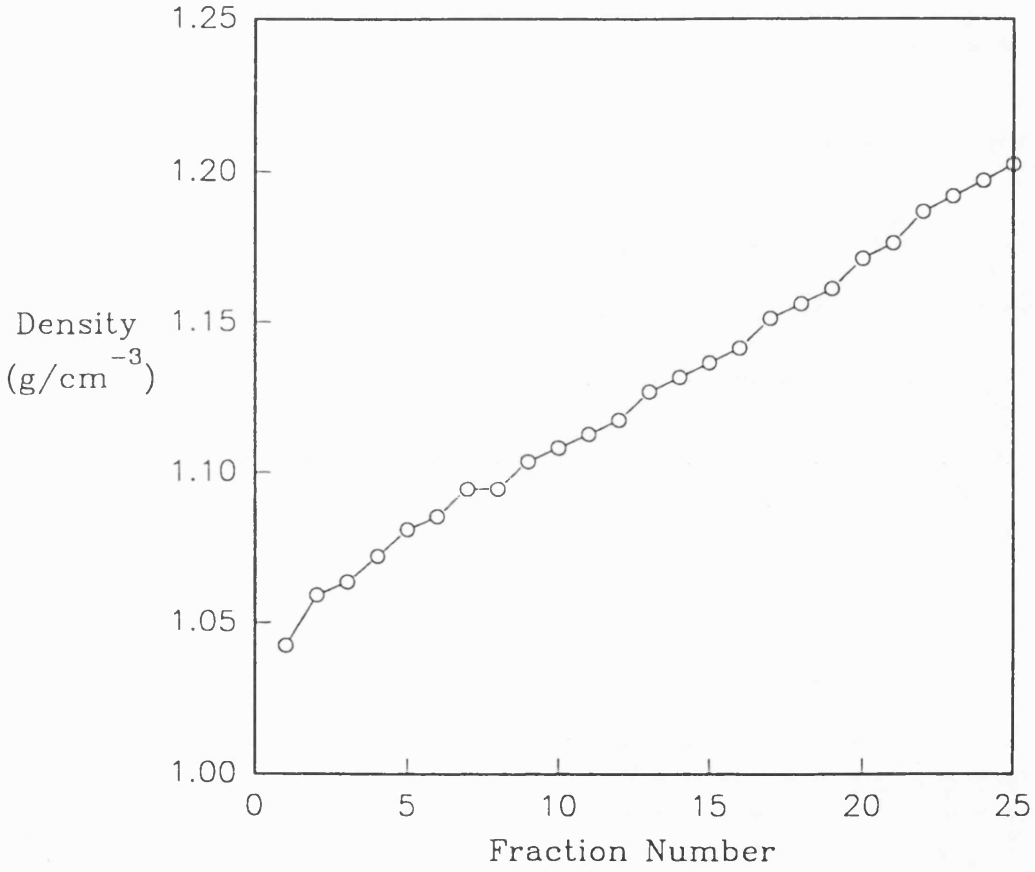


FIGURE 4.1: Density of fractionated sucrose fractions against fraction number

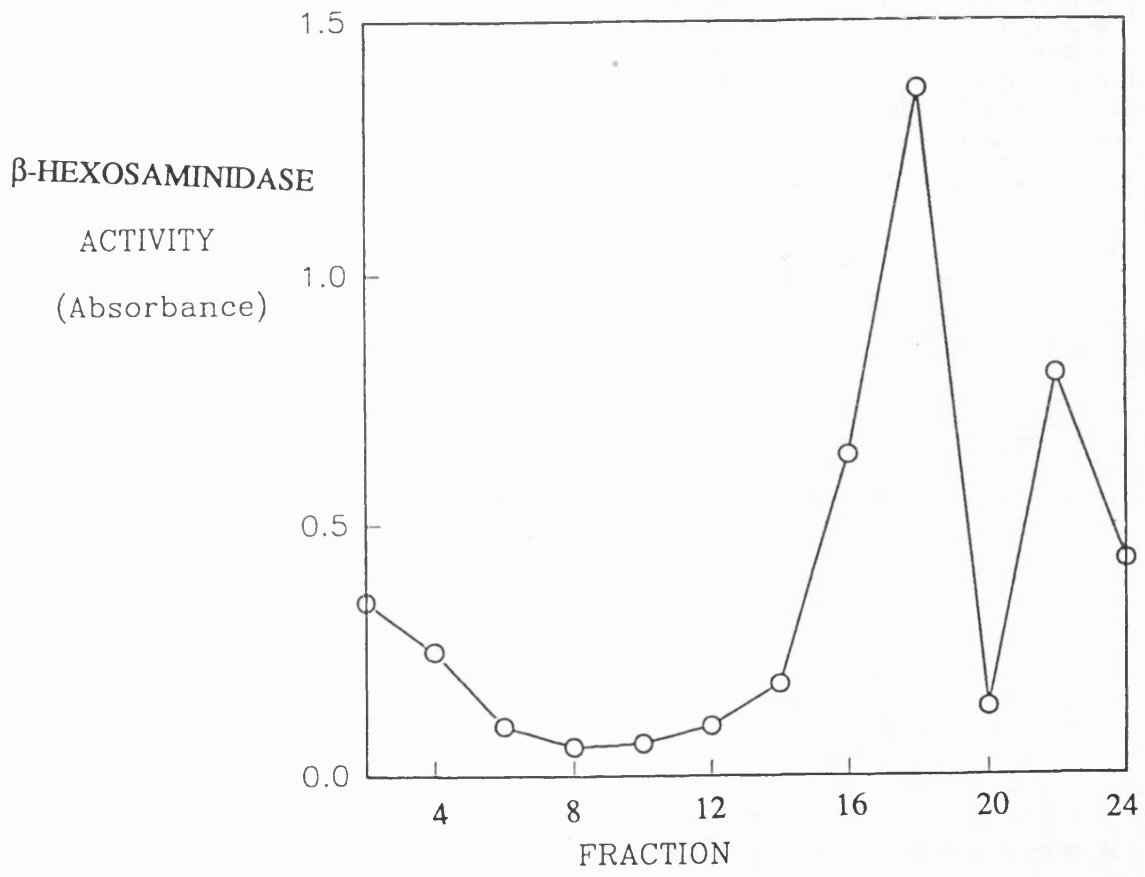


FIGURE 4.2a: β-Hexosaminidase activity present in fractions of density displayed

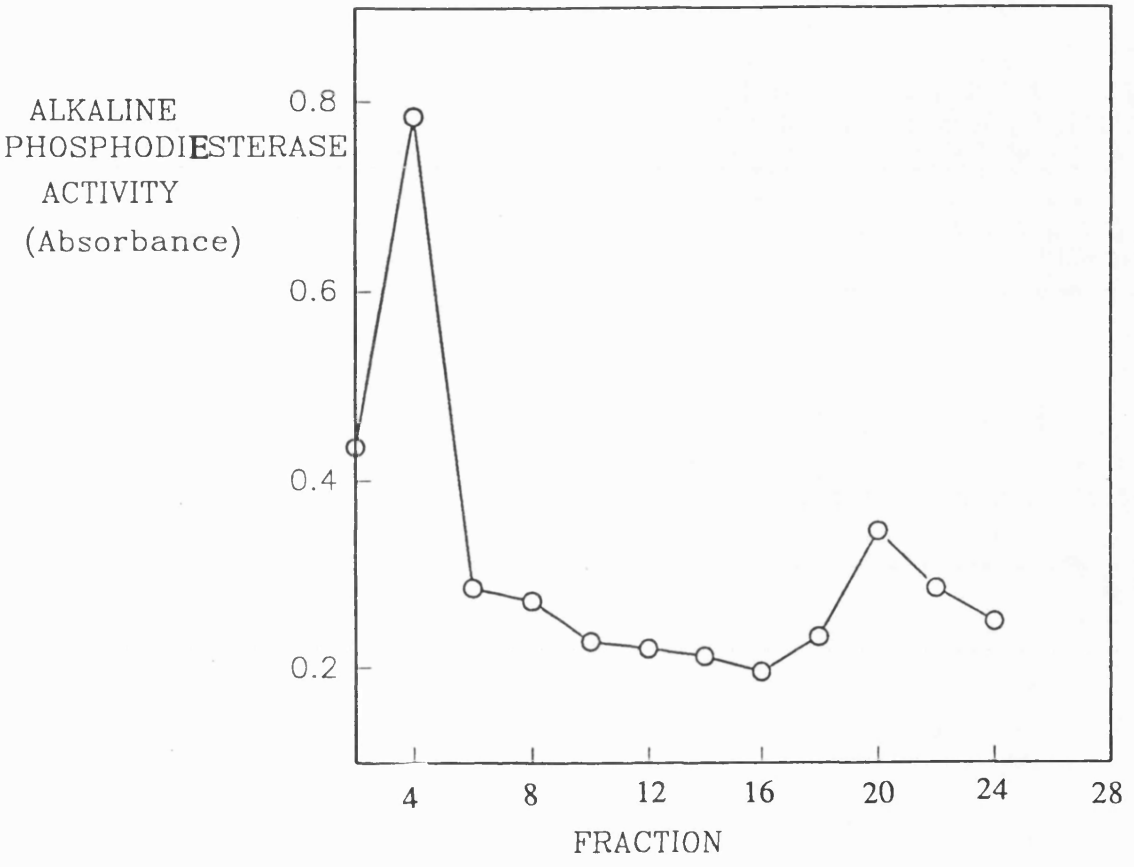


FIGURE 4.2b: Alkaline Phosphodiesterase I activity present in fractions of density displayed

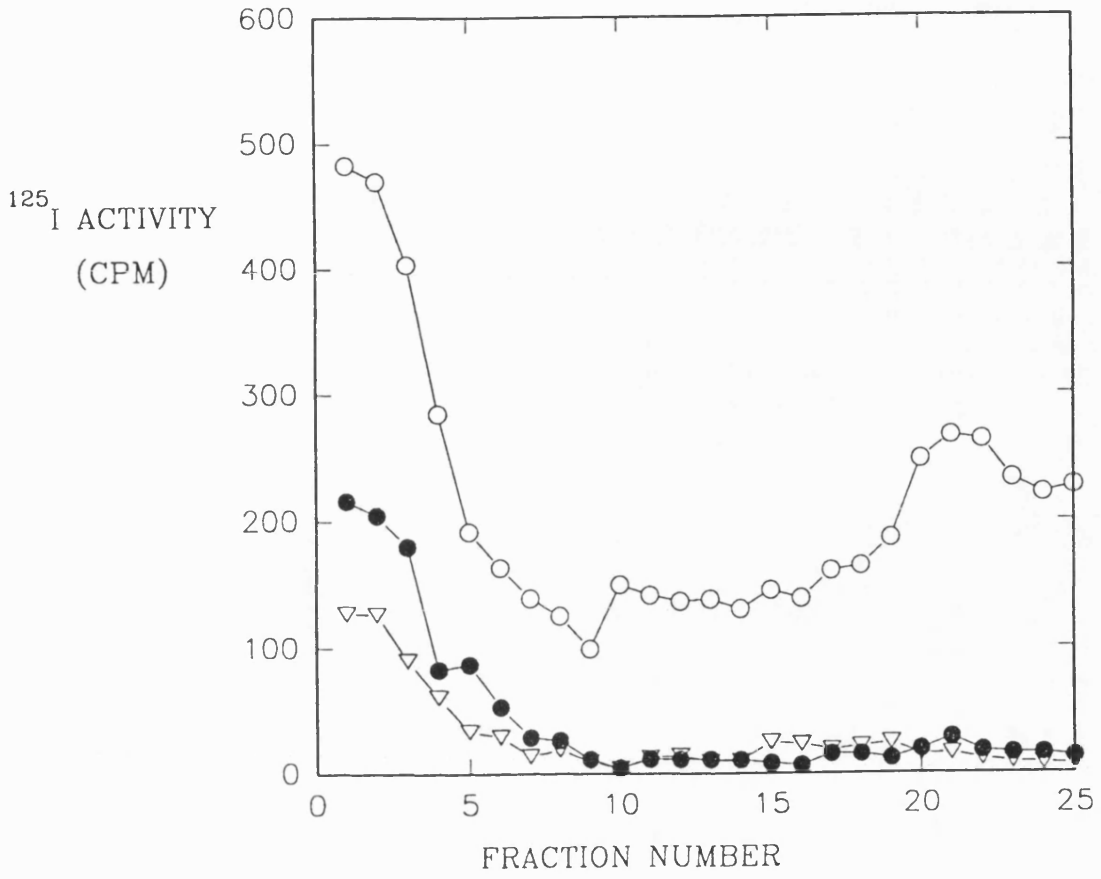


FIGURE 4.3: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH prior to homogenisation and fractionation. Total (○), non-specific (●) binding respectively and internalised ligand (▽).

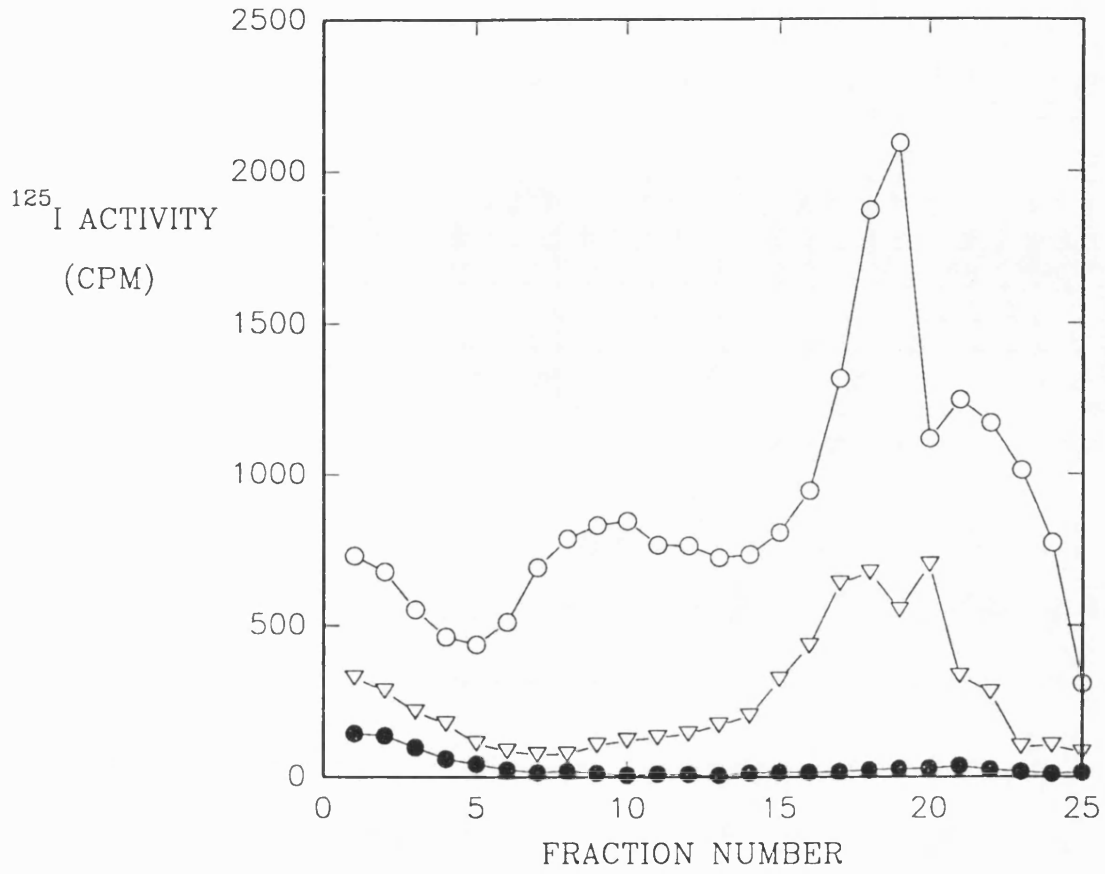


FIGURE 4.4: ^{125}I-activity associated with each sucrose fraction for cells incubated for 30 minutes at 37°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH prior to homogenisation and fractionation. Total (○), non-specific (●) binding respectively and internalised ligand (▽).

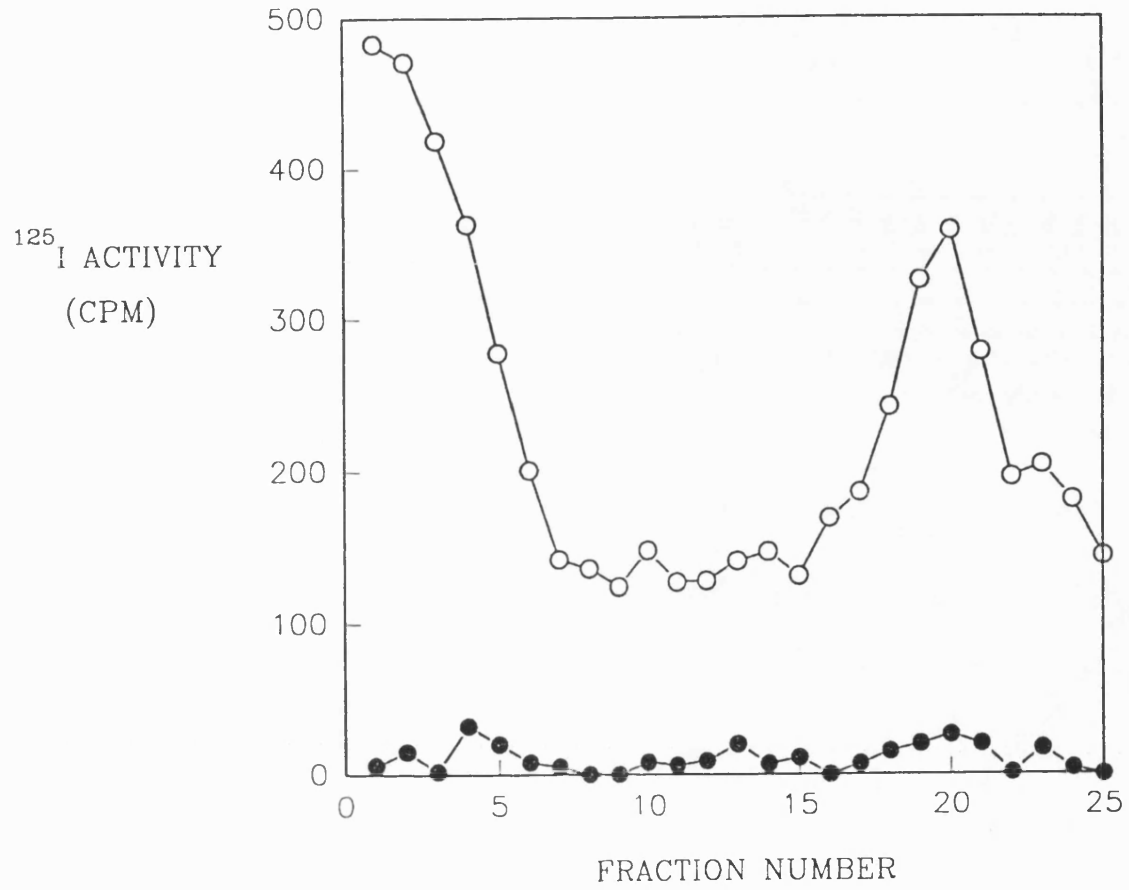


FIGURE 4.5a: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH prior to homogenisation and fractionation.Total binding (○) and internalised ligand (●).

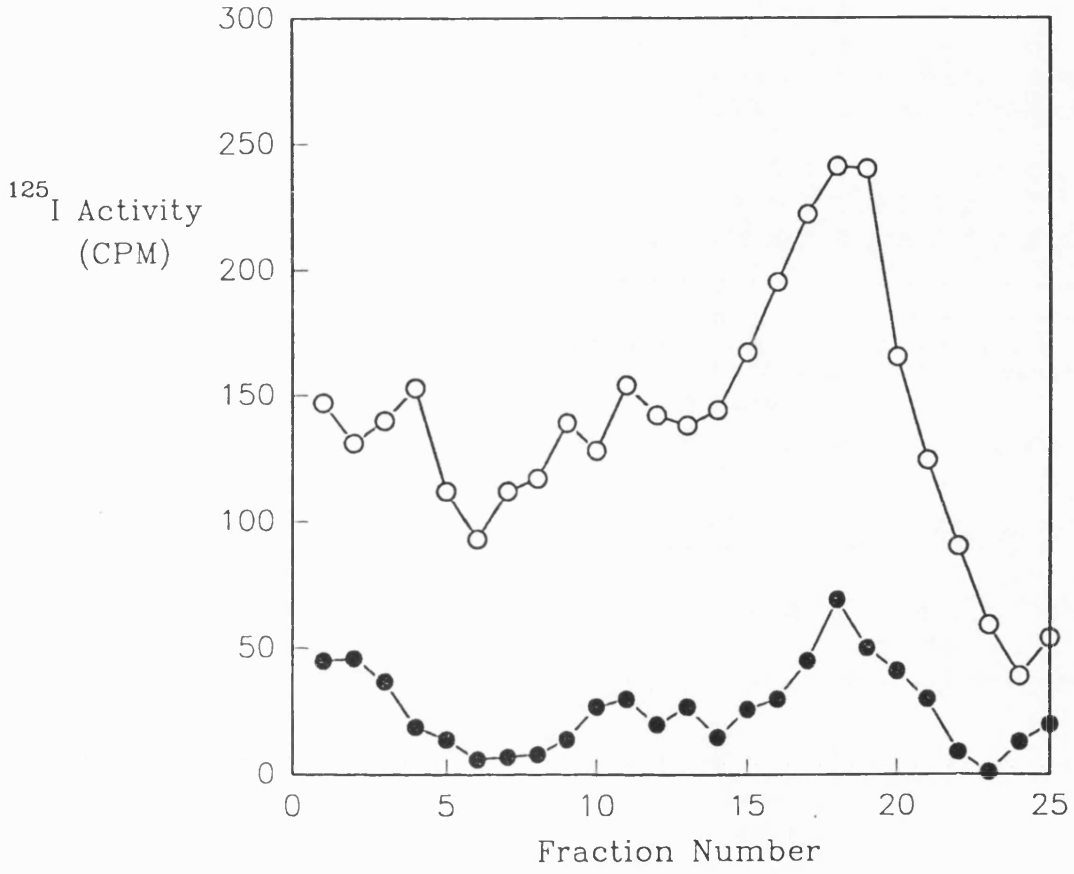


FIGURE 4.5b: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH, followed by incubation at 37 °C for 15 minutes prior to homogenisation and fractionation. Total binding (○) and internalised ligand (●).

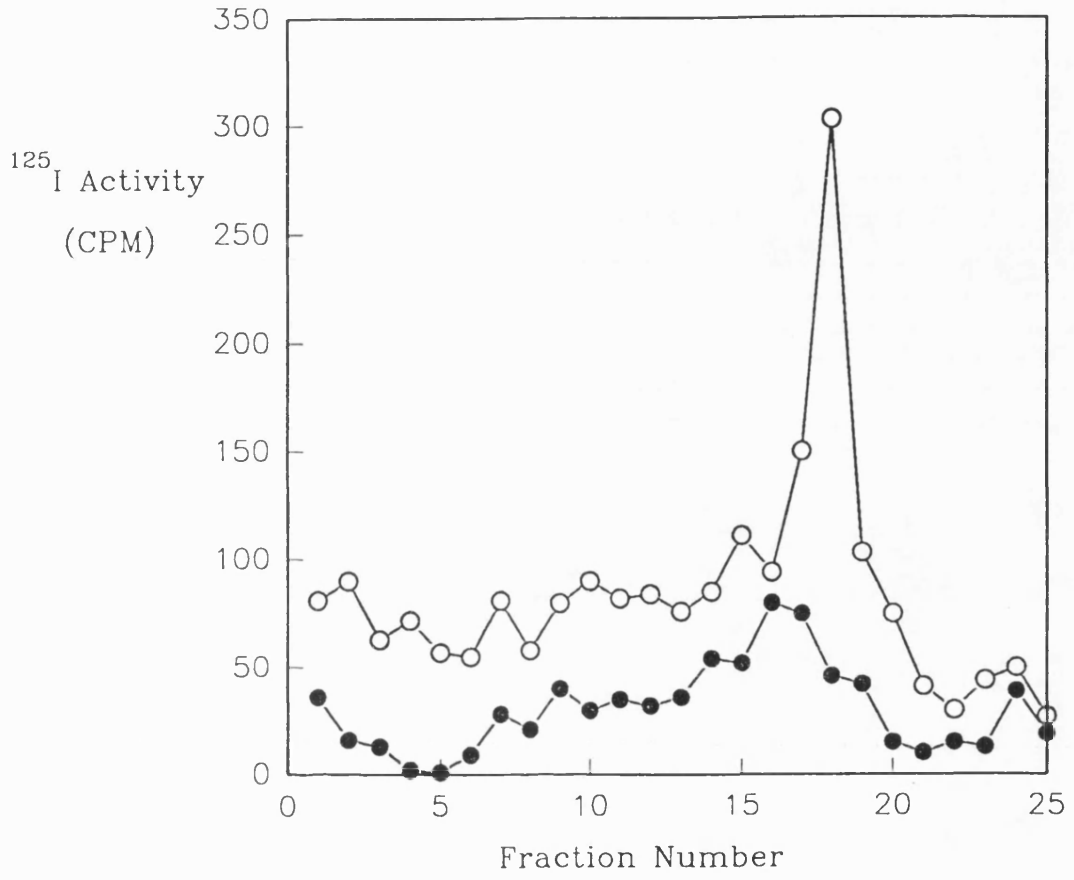


FIGURE 4.5c: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH, followed by incubation at 37° C for 30 minutes prior to homogenisation and fractionation. Total binding (○) and internalised ligand (●).

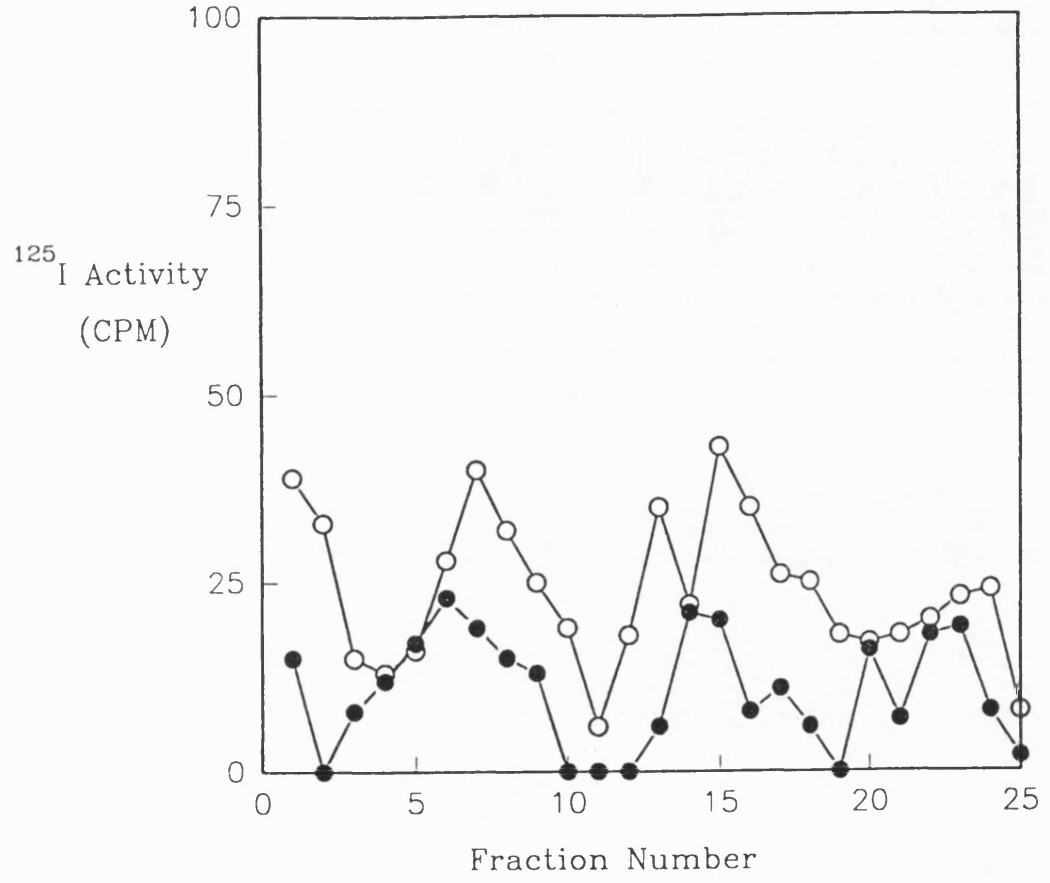


FIGURE 4.5d: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH, followed by incubation at 37 °C for 60 minutes prior to homogenisation and fractionation. Total binding (○) and internalised ligand (●).

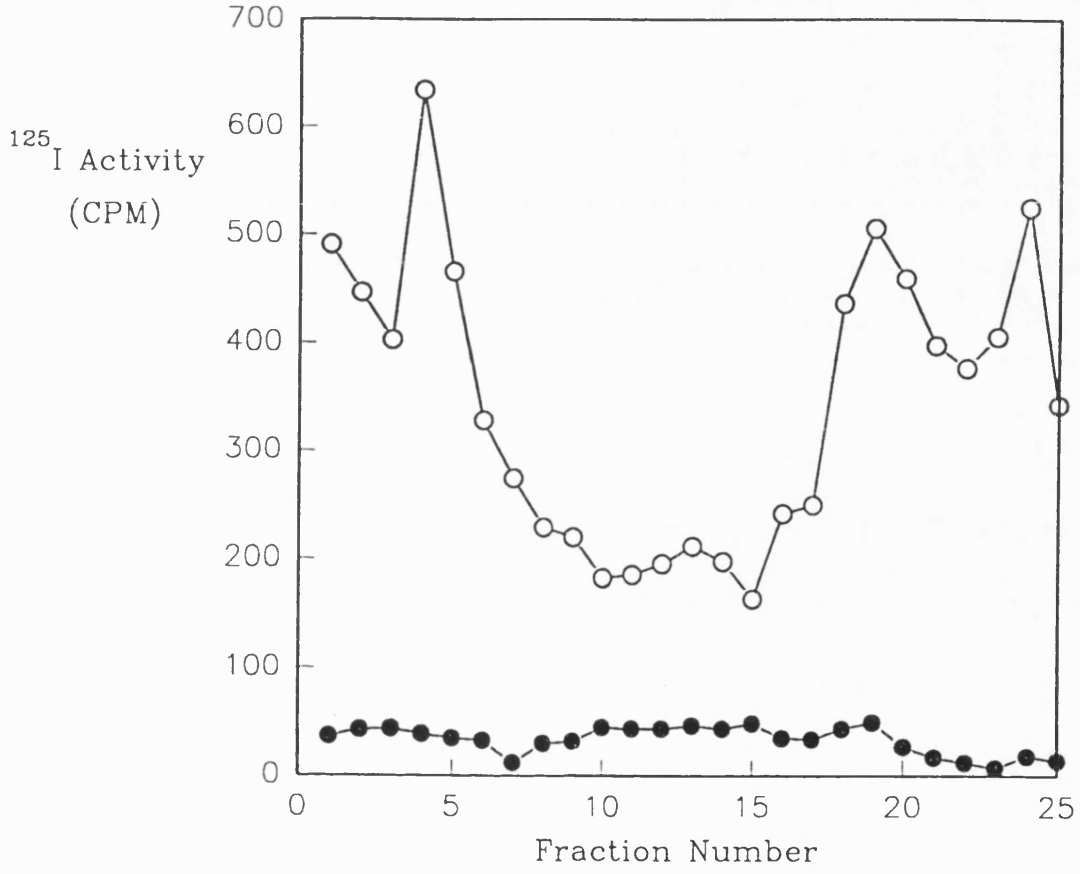


FIGURE 4.6a: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH prior to homogenisation and fractionation in the presence of 20mM NH_4Cl. Total binding (○) and internalised ligand (●).

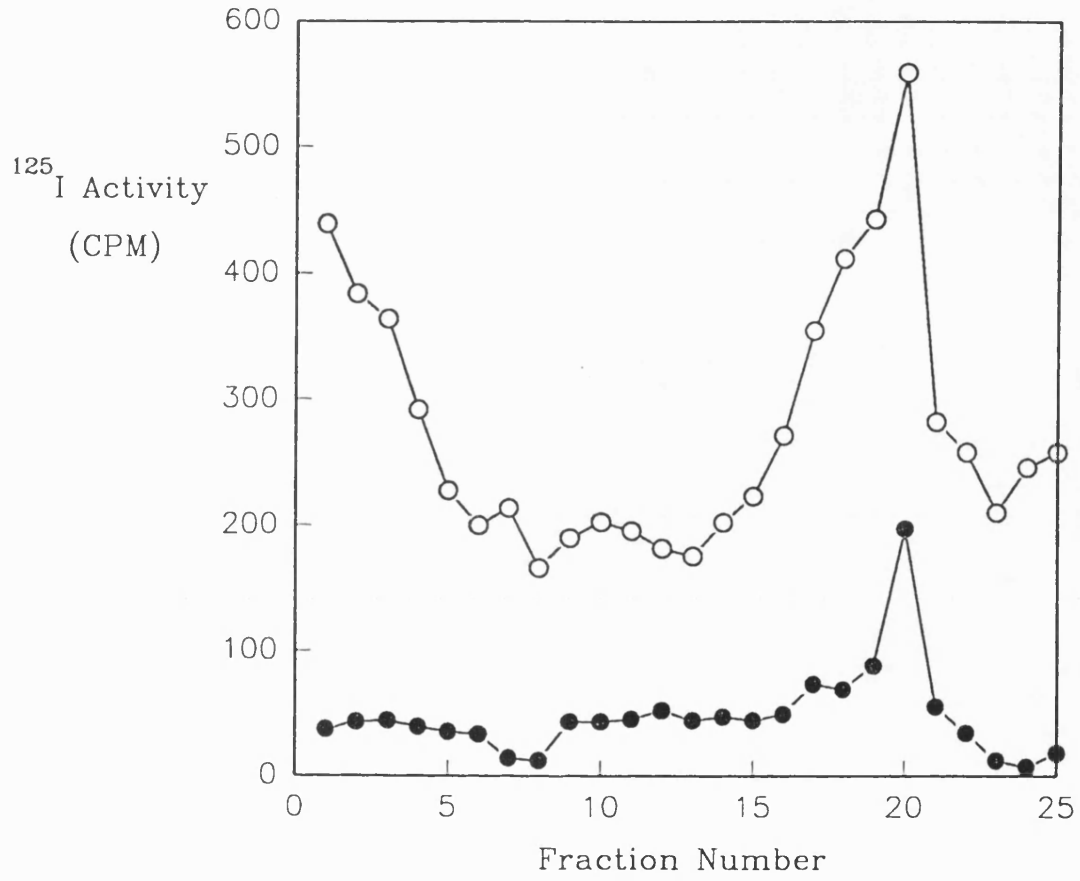


FIGURE 4.6b: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH, followed by incubation at 37 °C for 15 minutes prior to homogenisation and fractionation in the presence of 20mM NH_4Cl. Total binding (○) and internalised ligand (●).

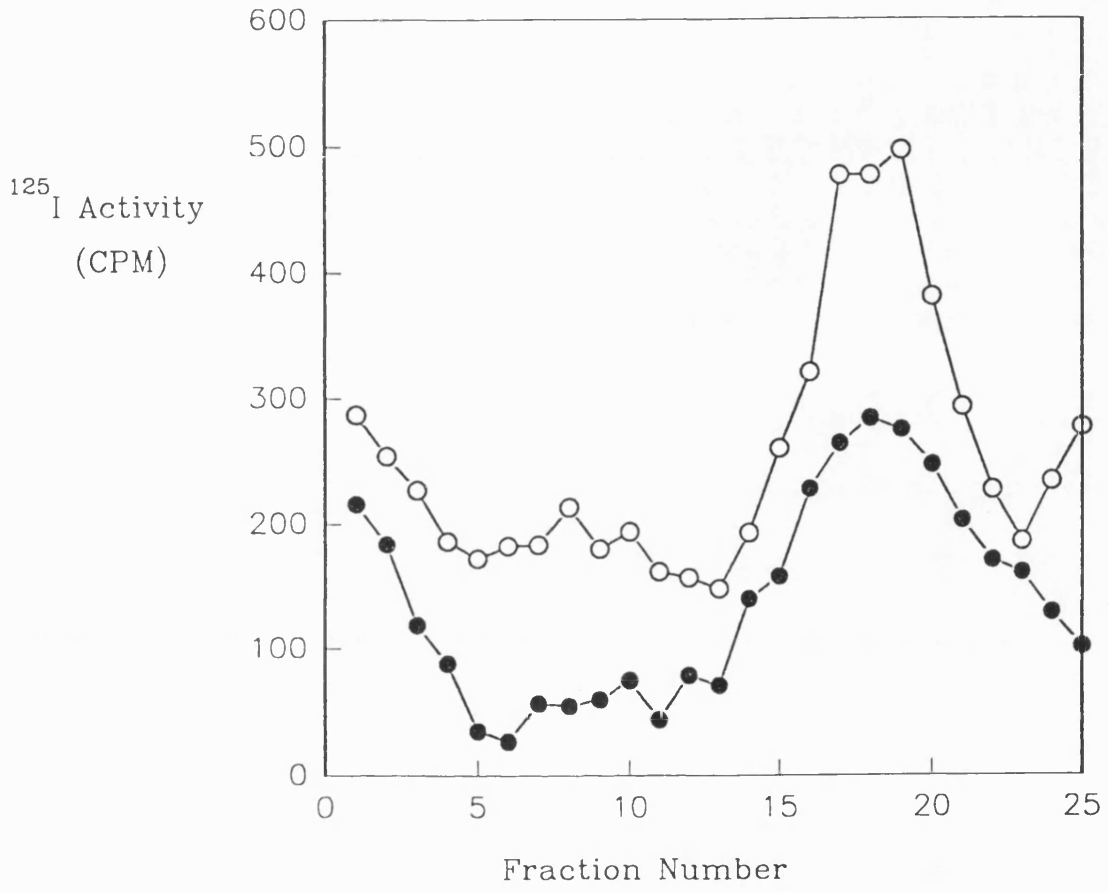


FIGURE 4.6c: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH, followed by incubation at 37° C for 30 minutes prior to homogenisation and fractionation in the presence of 20mM NH_4Cl. Total binding (○) and internalised ligand (●).

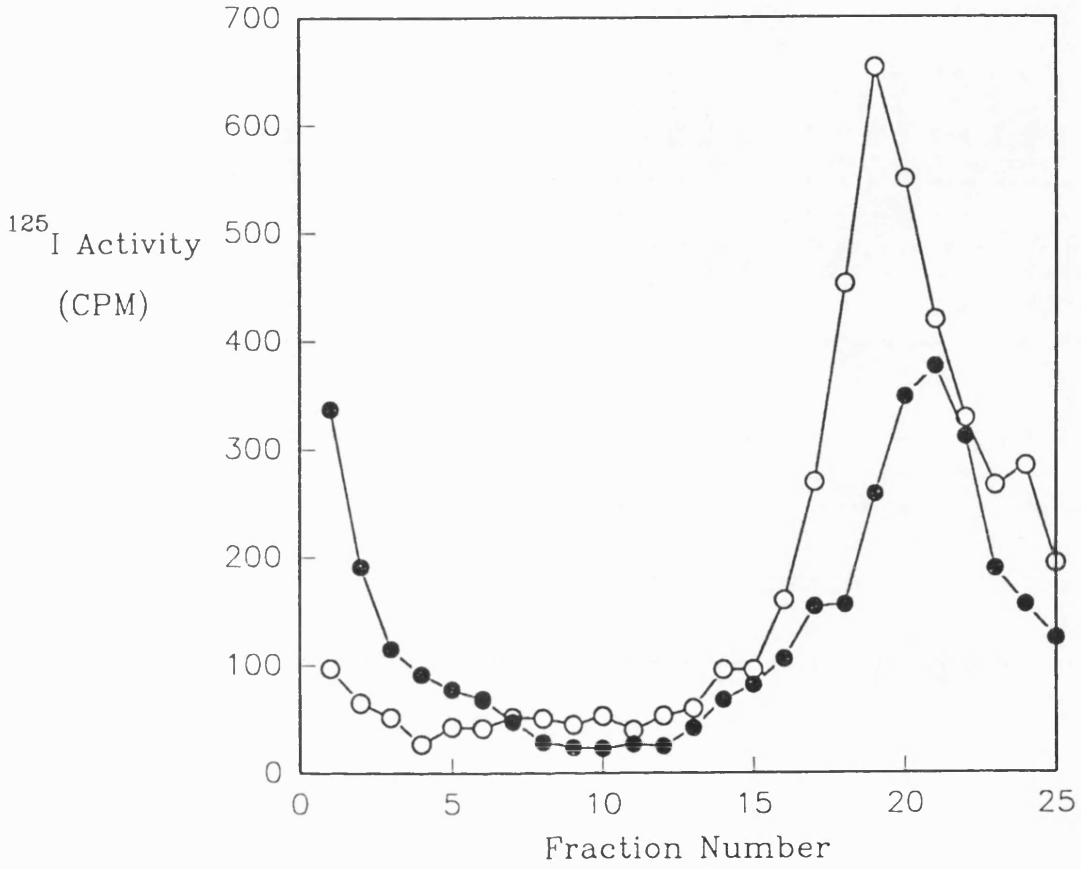


FIGURE 4.6d: ^{125}I-activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH, followed by incubation at 37 °C for 60 minutes prior to homogenisation and fractionation in the presence of 20mM NH_4Cl. Total binding ( ) and internalised ligand ( ).

4.4 DISCUSSION

The work studied in Chapter 3 dealt with the cellular binding and uptake of the α-MSH superpotent analogue, [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. Data obtained confirmed the specific binding of ligand at 4°C and 37°C. In addition, at 37°C, a marked amount of ligand was shown to be internalised, as was apparent from the acid wash data obtained. Moreover, the introduction of NH_4Cl, an exogenous amine, implied that once internalised the ligand makes its way to the lysosomal compartment prior to degradation or recycling.

In an attempt to observe that the ligand does travel to the lysosome, the subcellular fractionation of the B16 melanoma cells was performed. Due to the fact that very little work has been done concerning this area, it was necessary to start from the beginning and set up homogenisation, centrifugation and fractionation assays.

The initial step was to find a suitable homogenisation method suitable for disrupting the B16 melanoma cells; enough to allow separation of their subcellular components, but gentle enough to ensure the separated components remained intact. Too severe a disruption would cause breakage of the membranes of the internal components of the cells and would be of no use for these studies. A detailed explanation of the development of a suitable homogenisation protocol is given in section 2.2.4.1, page 51.

It was decided to concentrate on isolating the plasma membrane and lysosome. The reason being, at 4°C, from cell binding experiments described in chapter 3, binding occurs only at the cell surface without internalisation and hence, would only be observed in the plasma membrane fractions isolated. In comparison, at 37°C, it would be expected to observe increased activity in lysosomal fractions prior to the ligand leaving the cells. To define the individual components of plasma membrane and lysosome, enzyme marker assays were

individual components of plasma membrane and lysosome, enzyme marker assays were introduced. β-Hexosaminidase was chosen as the lysosomal marker and alkaline phosphodiesterase I as the plasma membrane marker (Muller, J.M. *et al;* 1985).

In order to determine if this sucrose gradient fractionation technique could discriminate between internalised ligand and extracellularly bound ligand, experiments were carried out at 4°C and 37°C. The data from such experiments did show that the iodinated ligand was eliminated by an acid wash for the 4°C experiment (figure 4.3), whereas, at 37°C (figure 4.4) ther was still ^{125}I activity associated with the heavier fractions of the sucrose gradient. The high level of ^{125}I activity present at low densities for the 4°C experiment could be explained by "free ^{125}I", that is, iodinated peptide which freed itself from the receptor; or it could be very small fragments of plasma membrane which exhibit low density.

Although these experiments indicated that a distinct separation of the plasma membrane and lysosome fractions could not be achieved using this procedure, they suggest that the fate of the ligands could be qualitatively determined. Therfore, a series of "pulse chase" time course experiments were designed with the major aim being to further confirm the internalisation of the ligand and trafficking to internal organelles including the lysosome.

The first series of time course experiments were carried out by bonding of the ligand for 2 hours at 4C, followed by 1, 15, 30 and 60 minutes at 37°C. For the cells incubated only at 4°C a peak of activity was observed between fractions 16 and 21. In the presence of an acid wash this was not observed indicating that that observed for total binding was associated with the plasma membrane. A similar observation was found for cells incubated at 37°C for 15 minutes after 2 hours at 4°C. This time however, not all the ^{125}I activity was removed when treated with an acid wash. A small "peak" of activity was observed

between fractions 16 and 20. For the third timepoint in this experiment (30 minutes at 37° C), a similar trace of activity was found as for the 15 minute time period. When the cells had been incubated for 60 minutes at 37°C, there was very little ^{125}I activity associated with the cells and no distinct areas containing higher levels of ligand were observed.

In comparison to this, the same experiment was performed but in the presence of 20mM NH_4Cl. The major difference was observed when the cells were incubated for 60 minutes at 37°C (figure 4.6d). A large area of ^{125}I activity was observed in fractions of higher density for both the acid washed data and total ^{125}I activity. Note, for the equivalent timepoint in the absence of NH_4Cl, no such peaks of activity were found. These data further imply that once the ligand has bound to the cell surface (plasma membrane), it moves inside the cell to intracellular organelles, probably the lysosome where degradation may occur.

In order to prove that degradation does occur, an experiment whereby the intactness of the ligand could be measured needs to be investigated. One way of doing this would be to prepare fragments of the ligand and determine their elution position using FPLC in conjunction with column chromatography techniques. Then, to determine if degradation has occurred, samples of the digested cells and the medium in which they have been incubating could be passed through the system and the elution of the fractions monitored. The appearance of degraded fragments would be observed if indeed, degradation is occurring. Panasci and his colleagues (1987), studying the metabolic fate of tritriated Ac-[Nle^4,D-Phe^7]α-$MSH_{4-11}NH_4$ with the F1 variant of B16 melanoma cells, made use of a 30cm column of Sephadex G-10 to monitor the degradation (Panasci, L.C. *et al;* 1987). They concluded the appearance of an intermediate degradation product at 4 hours of a cellular sample and furthermore, analysis of a 2 hour dissociation of a 30 minute

cellular sample revealed that the majority of the radioactivity eluted in the same position as leucine.

In conclusion then it can be said that the results obtained from subcellular fractionation studies further confirm internalisation of the ligand. They also support the theory that the lysosome has a major role (possibly degradation) in the decrease of activity associated with the B16 melanoma cells after 30 minutes.

CHAPTER 5

The Effect of Size of Ligand on the Internalisation of Analogues into B16 Melanoma Cells.

5.1 INTRODUCTION

If peptides can be used to target drugs specifically to their site of action, it is not just enough for the hormone itself to reach its required site and be internalised in order to fulfil its role. A drug molecule must also be able to reach the cell site and be internalised. The previous two chapters concluded that the [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH analogue did bind specifically to an α-MSH receptor and was rapidly internalised into the B16 melanoma cells. Subcellular fractionation studies implied the ligand once within the cells, travelled to the lysosomal regions where upon it was degraded or recycled. However, [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH is a small molecule and it would be of great interest to study larger ligands and observe how they bind, and/or are internalised into the B16 melanoma cells. To do this, N$^{\alpha}$-biotin-[Nle^{4},D-Phe^{7}]α-MSH and streptavidin-biotin-[Nle^{4},D-Phe]α-MSH were studied. These are two ligands of increasing molecular weight respectively and could be used as a "mimic" to a drug attached to the parent Ac-[Nle^{4},D-Phe^{7}]α-MSH analogue.

5.2 METHODS

These are described in detail in Chapter 2.

5.3 RESULTS

5.3.1 Internalisation of N$^{\alpha}$-Biotin-[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH into B16 Melanoma Cells at 37°C

N$^{\alpha}$-Biotin-[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH (0.1nM), was bound to 5 × 10^{5} B16 melanoma cells in a 24-well tissue culture plate for 2 hours at 4°C in the absence or presence of 1000-fold non-iodinated ligand. Unbound ligand was then removed and the cells were subjected to a second incubation at 37°C for various periods of time. The amount of activity associated with the cells specifically, non-specifically and internally was monitored as for [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH as reported in Chapter 3. As normal, the data displayed is the mean and standard deviation of four wells.

Figure 5.1 on page 114, represents the "pulse chase" internalisation of N$^{\alpha}$-Biotin-[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH. Specific binding was found to decrease gradually over the 90 minute period of incubation at 37°C, as was found with [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH (The data for which is as displayed in figure 5.7, page 120). In agreement with the [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH, the amount of internalised ligand increased to a maximum at 30 minutes and then decreased gradually over the remaining 60 minutes of the experiment. It must be noted that a lesser amount of N$^{\alpha}$-biotin-[Nle^{4},D-Phe^{7}]α-MSH appeared to degrade or leave the cells in comparison to the [Nle^{4},D-Phe^{7}]α-MSH ligand.

The experiment was repeated in the presence of 20mM NH_4Cl to observe the effect of the exogenous amine on the internalisation rate of the larger ligand. It was of interest to determine if the same effect would be apparent as was for the [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. Figures 5.2 and 5.8 (pages 115 and 121 respectively) display the data obtained for each ligand. A similar trend was obtained for the N$^{\alpha}$-biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH has had been obtained for [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. Specific binding gradually decreased throughout the 37°C incubation, whereas, the internalised ligand was found to increase over the same time period. However, for the N$^{\alpha}$-biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH, the amount of ligand associated with the cells was still increasing at 90 minutes and in fact 66% of that associated specifically was internally associated. This was in comparison to almost 100% of the [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH being internalised at the equivalent time period, that is, at 90 minutes all ligand specifically bound was internalised. These results would tend to suggest internalisation does occur for the larger ligand and degradation or recycling also appears to happen, but at a slower rate.

5.3.2 Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 Melanoma Cells at 37°C

The addition of streptavidin to the N$^{\alpha}$-biotin-[Nle^4,D-Phe^7]α-MSH ligand to increase the size of the ligand even further was next studied. The results obtained regarding the time course internalisation of the streptavidin ligand are shown in figures 5.3 and 5.4, on pages 116 and 117 in the absence and presence of 20mM NH_4Cl respectively.

The trend of binding and internalisation varies from that obtained for both the [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH and biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH for this analogue.

As figure 5.3 shows the specific binding of the streptavidin-biotin-[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}] α-MSH appears to decrease quite rapidly. In addition, the ligand although internalised, does so more slowly than for the previous ligands tested. Even at 90 minutes the ligand appeared to still be internalising. In other words, no maximum point of intracellular accumulation at 30 minutes (as had been apparent for the other ligands) was found to occur. Due to this observation, a further experiment was carried out in which the final time point for the measurement of internalisation was extended from 90 minutes to 240 minutes for the 37°C incubation. The data from this experiment is displayed in figures 5.5 and 5.6 on page 118 and 119 respectively. This time the familiar pattern of increasing internalisation to a specific time point, followed by a gradual decrease to the end of the experiment is observed. For streptavidin-biotin-[Nle^{4},D-Phe^{7}]α-MSH, the maximum amount of ligand within the cells was apparent at 90 minutes, followed by a gradual decline to the end of the 240 minute period.

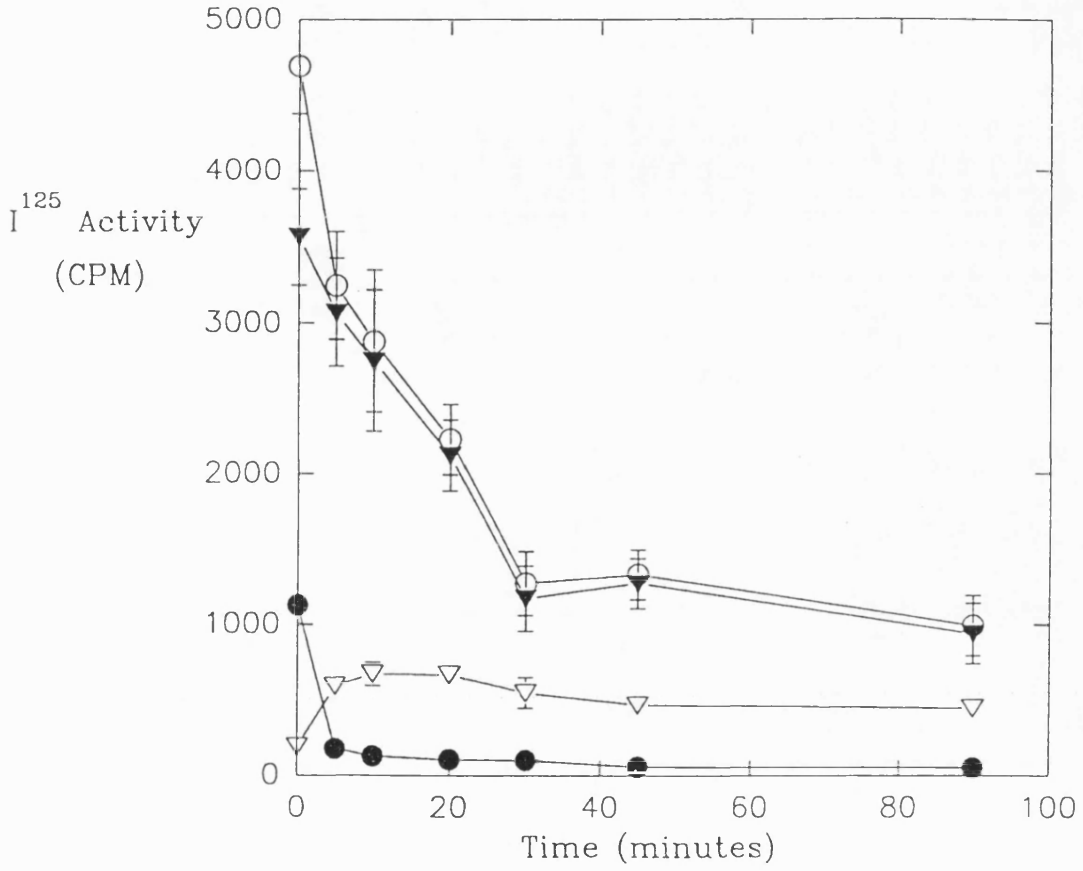


FIGURE 5.1: Time dependence on the internalisation of N^{α}-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM N^{α}-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

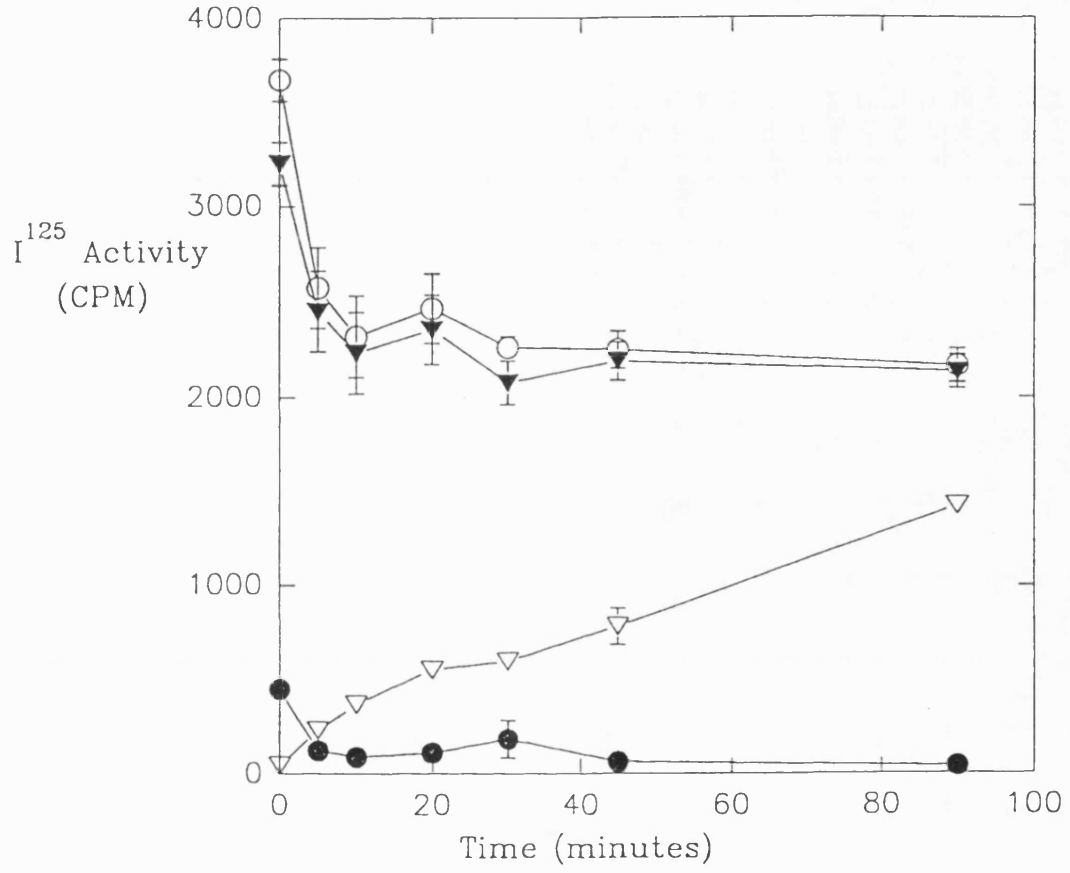


FIGURE 5.2: Time dependence on the internalisation of N^{α}-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM N^{α}-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the presence of 20mM NH_4Cl. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

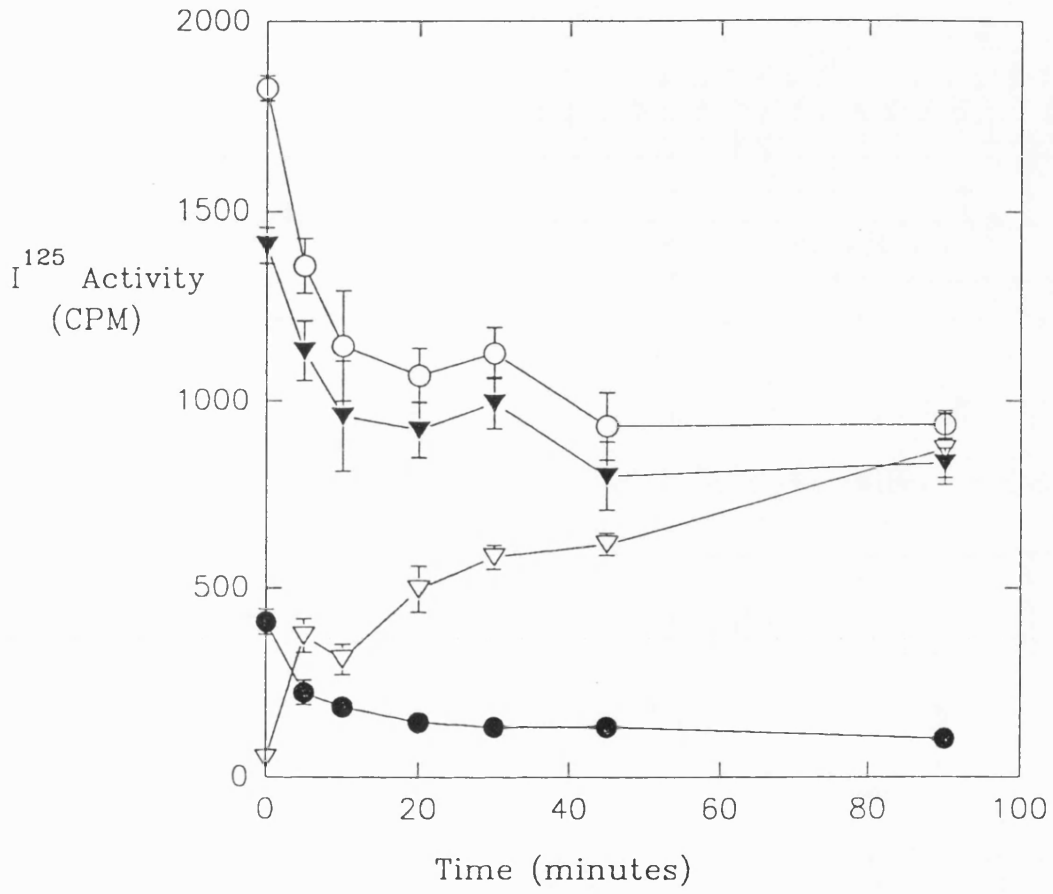


FIGURE 5.3: Time dependence on the internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

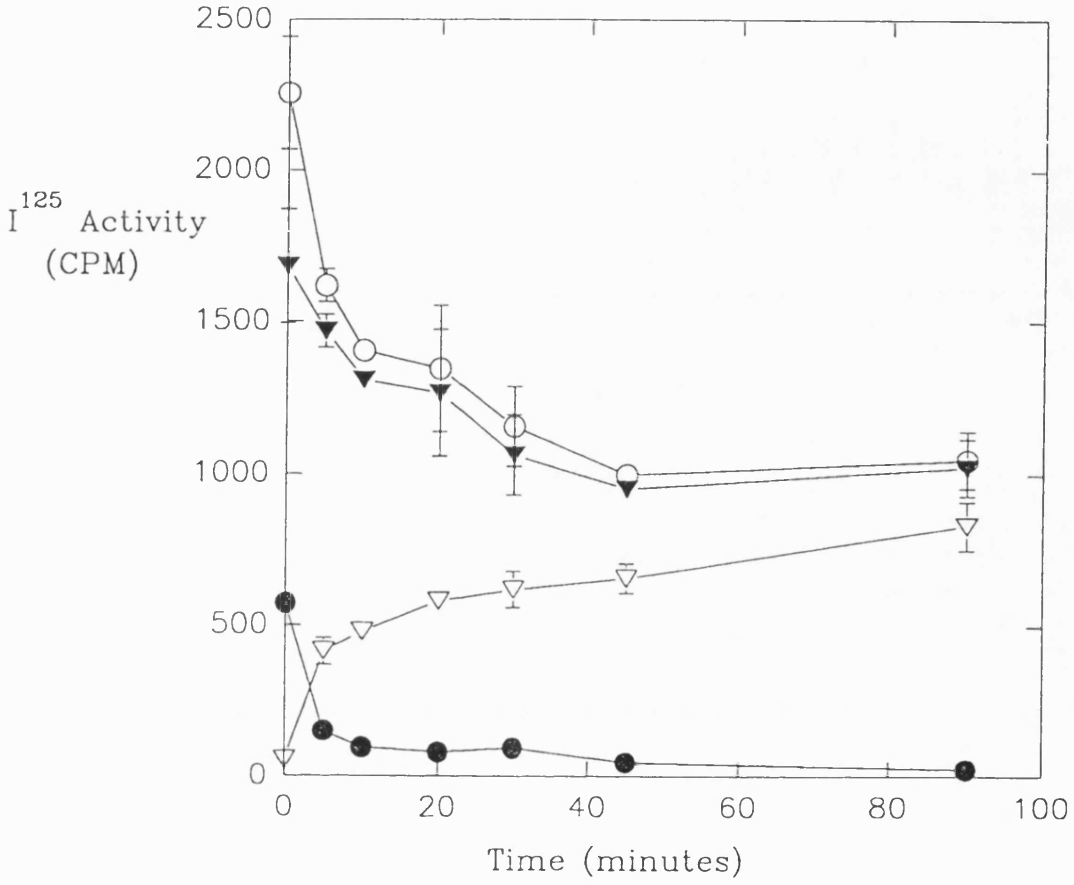


FIGURE 5.4: Time dependence on the internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the presence of 20mM NH_4Cl. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

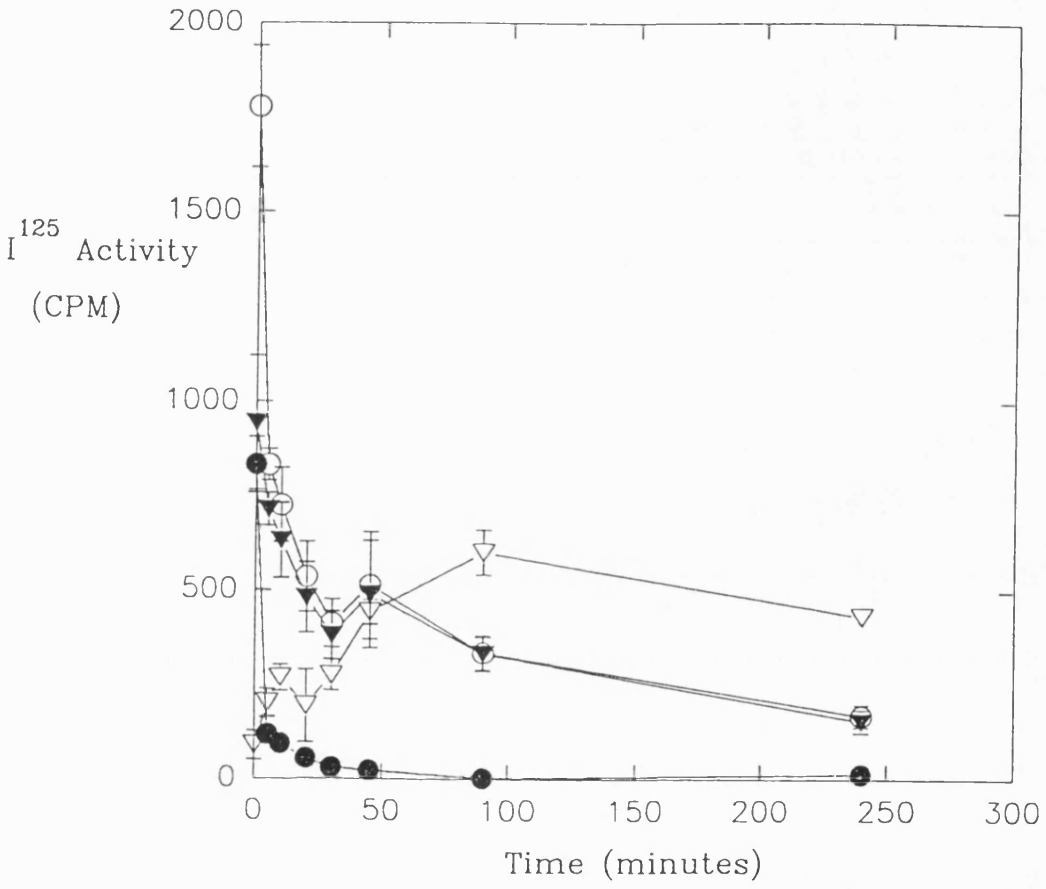


FIGURE 5.5: Time dependence on the internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 240 minute period. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

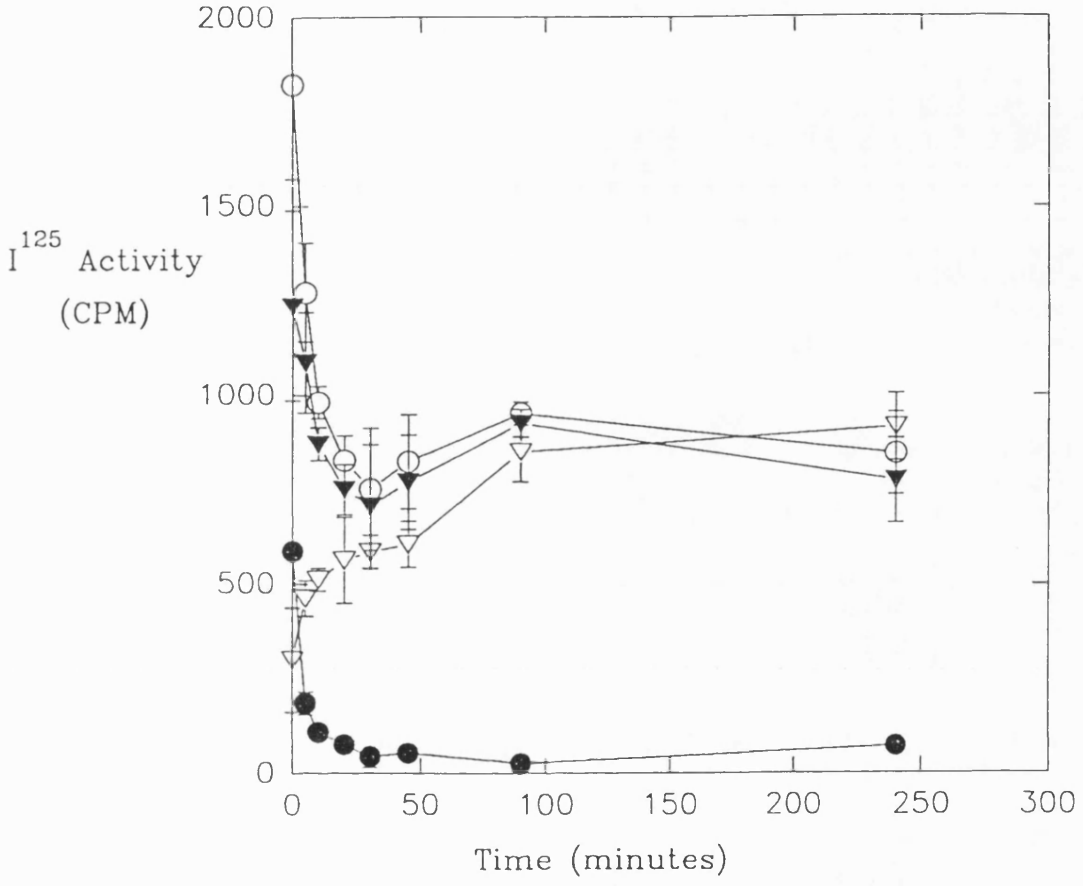


FIGURE 5.6: Time dependence on the internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 240 minute period in the presence of 20mM NH_4Cl. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

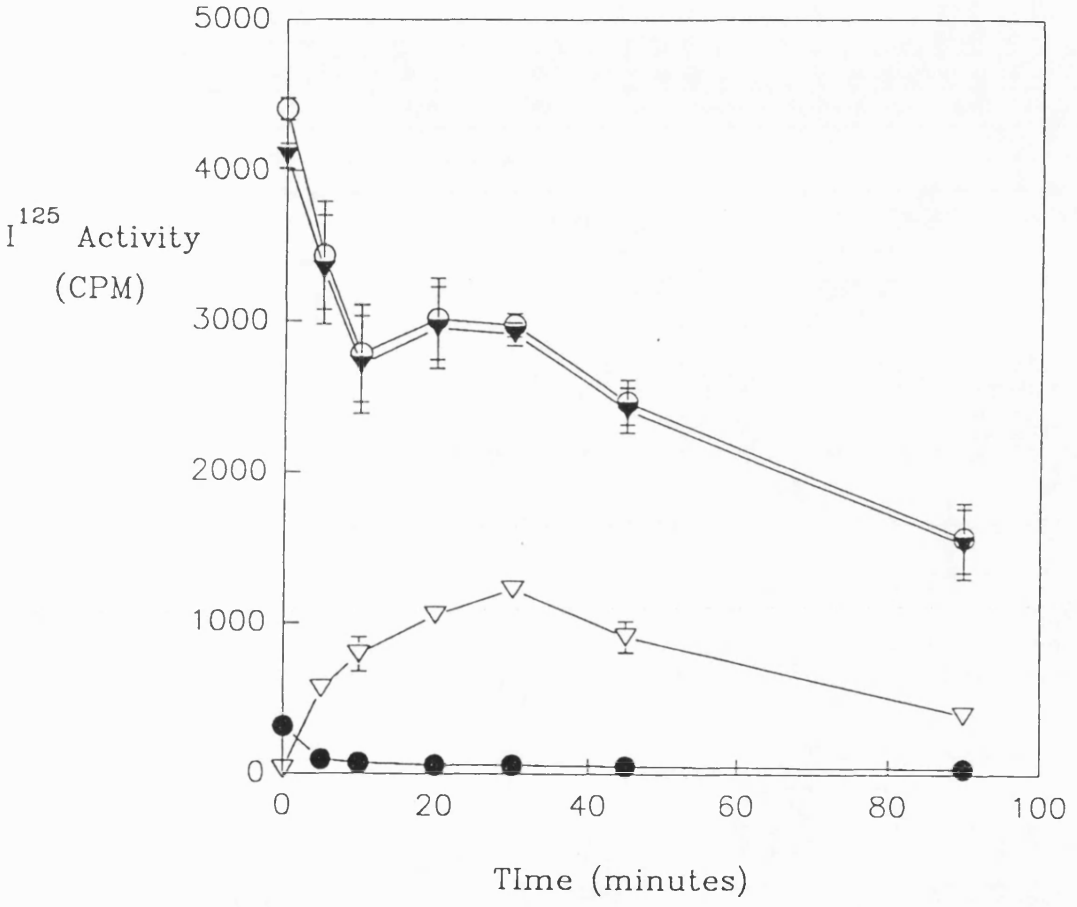


FIGURE 5.7: Time dependence on the internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

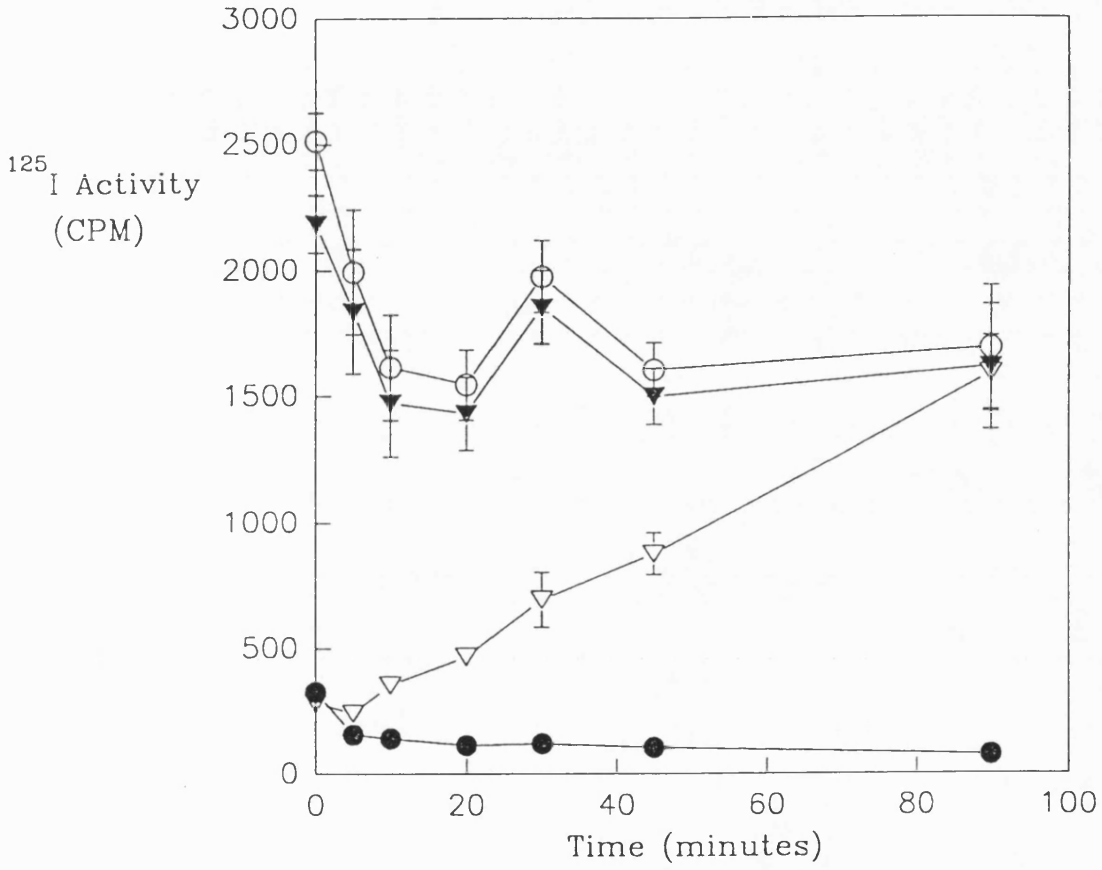


FIGURE 5.8: Time dependence on the internalisation of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH into B16 melanoma cells after binding of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the cell surface for 2 hours at 4°C, followed by incubation at 37°C over a 90 minute period in the presence of 20mM NH_4Cl. Total (○), specific (▼), non-specific (●) binding respectively and internalised ligand (▽).

5.4 DISCUSSION

As mentioned in the introduction of this chapter, it is not just enough for the Ac-[Nle^4,D-Phe^7]α-MSH to be shown to be internalised via the α-MSH receptor of B16 melanoma cells, if such a system has the potential in drug targeting of cytotoxic drugs to their specific sites of action. The drugs which would be required are of much larger molecular weight and without a comparison with larger molecules, no direct evidence that the system proposed in this study would be of any use whatsoever. Not only that but it may be necessary for these larger drugs to be internalised to actually have the desired effect. Hence, it was necessary to try and "mimic" attachment of a drug to the parent analogue, Ac-[Nle^4,D-Phe^7]α-MSH. Unfortunately, no such drug-peptide molecules were available for use at the time, however, the use of the analogues N$^\alpha$-biotin-[Nle^4,D-Phe^7]α-MSH and streptavidin-biotin-[Nle^4,D-Phe^7]α-MSH were introduced.

The data obtained from the cell well experiments suggest that internalisation of both the larger analogues was found to occur. This appeared to be in an efficient manner. Specific binding occurred for both analogues, although that for the streptavidin-biotin-[Nle^4,D-Phe^7]α-MSH appeared smaller than for the other two analogues. This was most probably due to the size of the added streptavidin portion which may interfere with the ability of the peptide to bind with the α-MSH receptor and be subsequently internalised. The acid wash results did show confirmation of internalisation for both the N$^\alpha$-biotin-[Nle^4,D-Phe^7]α-MSH and streptavidin-biotin-[Nle^4,D-Phe^7]α-MSH ligands. For both however, the rate of internalisation was found to be less than for [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. As far as streptavidin-biotin-[Nle^4,D-Phe^7]α-MSH was concerned a longer time period of 240

minutes, compared with the normal 90 minutes, was needed to observe the familiar pattern of internalisation.

In conclusion, it can be said that internalisation of the larger peptide molecules was indeed found to occur. Even though the system may not be as efficient as for smaller analogues, the fact that the larger molecules appeared to move inside the cells after their initial surface binding, indicates the feasibility of the use of the α-MSH receptor as a means of targeting drugs too and inside the melanoma cells. In fact, Richards, A.C. (1992), working in the same laboratory at the University of Bath, has confirmed the internalisation of a methotrexate-conjugate into B16 melanoma cells.

As far as what happened to the ligands once they were internalised, the experiments containing NH_4Cl (the exogenous amine which raises the pH of the lysosomes, hence, resulting in no degradation) were again performed for these larger sized molecules. The results obtained implied that once the ligand found itself inside the cells, they behaved in a manner similar to Ac-[Nle^4,D-Phe^7]α-MSH. That is to say, no decrease after 30 minutes and after 90 minutes for N^{α}-biotin-[Nle^4,D-Phe^7]α-MSH and streptavidin-biotin-[Nle^4,D-Phe^7]α-MSH respectively, was observed, when 20mM NH_4Cl was present in the cell system. Again, these results tend to favour the conclusion that the decrease found to occur after a certain time period could be due to degradation of the internalised ligand, or receptor-ligand complex, in the lysosomal compartment of the cells. As was discussed in chapter 4 when dealing with the fate of Ac-[Nle^4,D-Phe^7]α-MSH after its internalisation, a further set of experiments are needed. These would be designed in such a way as to determine the degraded fragments of the analogues. Initially a series of fragments likely to occur once the ligand has undergone degradation would be prepared using peptide synthesis techniques. Following iodination of these fragments they would then be passed through a column and their elution time noted. Then to determine the degradation

products from the cell system, the cell homogenate, and/or the medium the cells were incubated in would be passed through the same column and the elution times of products monitored.

Overall, as far as the larger molecules and their behaviour is concerned with the B16 murine melanoma cells studied here, it is suffice to say that internalisation does occur, even though possibly at a reduced rate as the size increases. Hence, the future potential of the use of this peptide targeting system of α-MSH analogues would appear to be favourably indicated by the results obtained during the course of this study.

CHAPTER 6

DISCUSSION

The major aim of drug targeting, or site specific drug delivery, is to selectively deliver therapeutic agents to the desired site of action. If this criteria is fulfilled then, not only will the therapeutic effectiveness of the drug be increased dramatically, but also, there will be a vast decrease in the toxic side effects often associated with drugs. Unfortunately, such a process is not simple due to the complexities of the human body. More often than not, drugs prescribed for one disease can, due to lack of specificity, cause damage to normal cells or tissues, and in effect may cause more harm then there already is. Due to this inability of getting drugs to the site of disease accurately and without harming surrounding areas, the concept of site specific drug delivery was introduced.

The basic principle of site specific drug delivery is to find some way of getting the drug from its site of entry into the body, to the diseased area and nowhere else within the body. Such an achievement would not only cancel out damaging undiseased cells/tissues, but, would also lengthen the life time of the drug, or in other words, decrease the degradation and premature excretion of the drug before it has carried out its desired effect.

Although a major hurdle would have been overcome if one could successfully do this, and actually transport the drug to the site of action only and in a therapeutic form, there is then the question of how does the drug actually carry out its action? For the majority of drugs, this does not just involve transporting the drug to the damaged cell, but, it usually relies on the drug entering the cell and being capable of giving the desired effect once inside the cell. Thus it is very much apparent the complexities involved in drug targeting. Not only

does the human body cause severe problems in allowing the drug to reach its "goal", the diseased cells, but, then the cells themselves must contain certain molecular moieties which will allow the entry of the drug or drug/carrier.

As far as peptides are concerned, such a process is carried out by a receptor. Receptors are functionally linked to an effector system that is triggered upon agonist (ligand) binding. Being in most instances a membranal system, we must consider the receptor-effector system as a transmembrane signalling system, where the receptor faces the outside of the cell and the effector faces the intracellular space. The number of carrier-ligand receptors expressed on the target cell as well as the distribution of such receptors in other cells and tissues are important parameters which need to be considered in selecting a particular ligand as a carrier (Shen, W-C, *et al;* 1992). In general, the pharmacological effects of drugs are determined by many factors including:-

1. Receptor binding.
2. Cellular internalisation.
3. Intracellular sorting and targeting.
4. Intracellular transport.

The ligand predominantly employed in this study was the superpotent analogue of α-MSH, [Nle^4,D-Phe^7]α-MSH. It was radiolabelled and the mono-iodinated form used in all experiments performed in this study. The choice of cellular system used in conjunction with this analogue, were the B16 murine melanoma cell line. It had been established by Solca, F and his colleagues (1989), that a α-MSH receptor does exist. They synthesised a potent α-MSH photolabel, ^{125}I-Naps-MSH and applied it to mouse and melanoma cells; they characterised a 45kDa protein of B16-F1 cell membrane. Therefore, the system used

in this study was such that a receptor for α-MSH and its analogues was known to exist, and a suitable, superpotent probe, [Nle^4,D-Phe^7]α-MSH was available for use.

Before initiation of this study, it had already been established in our laboratory, that [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH displayed specific binding of K_d 0.37-0.87nM. It was also known that only 4000-5000 binding sites per B16 cell were available (Erskine, M.E; unpublished results). So although it had been confirmed that [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH elicits its biological action through binding to the MSH-membrane receptor, it was unknown whether or not internalisation of the receptor/ligand occurred. Hence, the initial experiments were designed so as to establish such a point.

It has been observed in a number of ligand-receptor systems that surface-bound radioligand can be dissociated by lowering extracellular pH. In these situations the fraction of total cell-associated binding removed by acid-exposure of cells is interpreted to represent surface-accessible binding. Hence, any activity associated with the cells after acid treatment represents internalised ligand. The validity of using reduced pH to dissociate ligand from receptors at the cell surface and thus distinguish surface-bound from internalised radioligand has been confirmed by Carpenter and Cohen (1976), by performing studies using quantitative electron microscopy radiography.

The acid wash procedure developed for the study of [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH internalisation into B16 melanoma cells, was 0.1M citrate buffer, consisting of citric acid, sodium chloride and 1M NaOH to give a working pH of 2.5. Work not reported in this study, but which was carried out, showed that a pH of 2.5 was necessary to remove cell surface bound ligand, both in the cell binding experiments and cell fractionation studies.

To prove the phenomenon of internalisation in the system chosen for this study, initial experiments were carried out whereby, binding of [^{125}I-Tyr^{2}-Nle^{4},D-Phe^{7}]α-MSH was allowed to bind to B16 melanoma cells either in the presence or absence of excess unlabelled ligand both at 4°C and 37°C. In addition, binding of the ligand followed by an acid wash was included. At 4°C, binding was shown to be increasing over a 4 hour period. The acid treated cells, on the other hand, displayed very little ^{125}I activity, suggesting no internalisation was occurring at this temperature. For the same experiment performed at 37°C, acid-resistant ^{125}I activity was apparent. The profile of ^{125}I activity over a 4 hour period, displayed maximum internalised ligand at 60 minutes followed by a gradual decrease. Unfortunately, no way of assaying the ^{125}I activity was available in order to determine the chemical state of the radioactivity. The decrease in internalised ligand after 60 minutes could be due to degradation. However, to confirm such a fact, chromatographic techniques are needed which can identify the [^{125}I-Tyr^{2}-Nle^{4},D-Phe^{7}]α-MSH in an intact state, and also fragments of the peptide.

Although it was not possible to ascertain whether the ^{125}I activity associated internally was associated with [^{125}I-Tyr^{2}-Nle^{4},D-Phe^{7}]α-MSH, or more likely, in a degraded form, some insight into what was happening after internalisation could be achieved by the lyosomotropic agent by increasing the pH of the endosome and lysosomal compartments, hence restricting lysosomal degradation and preventing vesicle fusion events in the endocytic pathway. Inclusion of 20mM NH_4Cl into the system had a very dramatic effect on the internalised ligand. No peak level of acid-resistant ^{125}I activity was observed. Instead, the amount of ^{125}I activity associated with the cells over a 4 hour period at 37° C, was shown to be continually increasing. At 4 hours, the amount of ligand (^{125}I activity) associated with the B16 cells, appeared to be internalised. The conclusions drawn from such a study are that degradation is the most probable reason for the decrease in ^{125}I activity within the cells after 60 minutes. By the inclusion of NH_4Cl, this decrease is not

present and since NH_4Cl is an inhibitor of lysosomal degradation, it would appear to be valid to suppose that degradation of the [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH is the main cause for the decrease in acid-resistant ^{125}I-activity associated with the B16 melanoma cells.

To summarise the results so far, it can be said that internalisation of [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH does indeed occur. Moreover, some insight into what may happen once internalised has been obtained with the work carried out in the presence of the exogenous amine, ammonium chloride.

Despite the fact that confirmation of internalisation has been determined, it would now be reasonable to surmise the use of the α-MSH receptor in the role of internalisation of the ligand. Therefore, a series of experiments were designed whereby the ligand was exposed to the cells for 2 hours at 4°C (i.e. no internalisation was permitted, only cell surface binding) and upon removal of excess iodinated ligand not bound to the cells, the cells were incubated at 37°C for further time periods. It was assumed that during the 4°C incubation, [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH was able to bind to the α-MSH receptor at the cell surface. Then during the 37°C incubation, this would be internalised via the α-MSH receptor. If internalisation was observed then it would be implied that the receptor was responsible for such a process and receptor-mediated endocytosis had occurred. In addition, such a set of experiments would illustrate the time-course of the internalisation process in more detail.

The outcome of these experiments suggested that the α-MSH receptor was employed in the transfer of externally bound ligand on the cell surface, to the inside of the cells. Internalised, or acid-resistant, ^{125}I activity was immediately present once the cells were transferred to 37°C. Moreover, a maximum amount of iodinated ligand was measured 30 minutes after the cells had been transferred to the 37°C incubator, longer periods of

incubation displayed decreased levels of acid-resistant ^{125}I activity with the cells. In conjunction with this observation, the amount of ligand bound specifically to the cells was seen to decrease gradually throughout the entire 90 minute period of the 37°C incubation. It can be suggested that, since the bound ligand decreases in parallel with an increase of ligand inside the cells, the iodinated ligand initially bound to the cell surface α-MSH receptor is transported into the cells at metabolic temperature (37°C).

As previously observed, there was a maximum amount of internalised activity observed, followed by a gradual decrease. So, as for the previous experiments, ammonium chloride was introduced into the system and again this "peak" of activity was avoided. Instead, the internalised activity continued to increase for the whole 90 minute period of the experiment. This further confirms that degradation occurs after the ligand is within the cells, and now, it would appear that it occurs 30 minutes after the ligand is internalised. So could the ligand be entering the cell via receptor-mediated endocytosis, making its way along the endocytic pathway to the lysosomal enzymes and subsequently degraded and expelled, resulting in a reduced amount of ligand appearing intracellularly?

In an attempt to answer this question, the inclusion of enzymes known to inhibit certain classes of lysosomal enzymes were introduced into the cell system. About fifty lysosomal enzymes are known and their properties are reviewed by Barrett (1984). There are basically four pathways involved in lysosomal metabolism: proteolytic, glycanolytic, nuclease and lipolytic pathways. The proteolytic and nuclease pathways start with enzymes specialised for action on the inner regions of the intact polymers that are their substrates, the endopeptidases and endonucleases, respectively. Early evidence of the role of lysosomes in the intracellular degradation of endogenous proteins came from the work of Dean, R.T. (1977), with liver cells, in which it was shown that pepstatin partially

inhibited the process. Leupeptin, a second protease inhibitor, is known to inhibit cysteine/serine proteases (Barrett, A.J.;1984).

With this knowledge, an attempt was made to observe if by adding pepstatin A or leupeptin to the experimental set-up, inhibition of degradation occurred. For both inhibitors, no observable increase in the intracellular ^{125}I activity, which would reflect an inhibition of protease degradation, was found to occur. It would appear then that the protease enzymes present in the lysosome are not responsible for the degradation of the ligand. However, it must be emphasised that a comparatively low concentration of the inhibitors were employed ($10\mu gml^{-1}$). Harford & Klausner (1987), for example in their attempt at studying receptor-mediated endocytosis in rat hepatocytes, treated cells with a 10-fold more concentrated solution of leupeptin. Using $100\mu gml^{-1}$ of leupeptin, they found an accumulation of radioactivity in the lysosomal region of the percoll gradient they employed in subcellular fractionation of their cells (Harford, J. & Klausner, R.D.; 1987). Unfortunately there was no time to repeat these experiments using a 10-fold increase in the protease inhibitors chosen here. So, although, the results obtained in this study suggest that the protease enzymes are not responsible for the degradation of the internalised [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH, in no way can this be accepted as the whole story.

Overall, as far as internalisation of the α-MSH analogue, [Nle^4,D-Phe^7]α-MSH is concerned, and, what happens to it once it has been internalised, the following facts have been determined:-

1. Internalisation of [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH does occur.
2. Maximum internalisation is seen to occur at 30 minutes, followed by a gradual decrease.

3. The cause for the decrease in acid-resistant ^{125}I activity has been shown to be linked with degradation of the ligand by inclusion of NH_4Cl into the cell system.

It must be further noted that an essential area to be examined as confirmation of these observations, is the identification of the degraded ligand. Although not reported here, an attempt at proving that degradation was happening was tested using the familiar trichloroacetic acid method. No success was achieved however. This was mainly due to the fact that the intact [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH, itself, was not precipitated by 10% TCA. Therefore, this method of confirming degradation could not be used. In addition, a very naive approach incorporating HPLC techniques as examined. Unfortunately, the background ^{125}I activity present on the HPLC equipment available for analysis of the test samples was much greater than the level of ^{125}I activity within the cells or incubation media from the cells. In respect of these initial attempts at identifying degradation products of [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH, much scope is available for an independent study. One pathway possibly worth examination is one already started by Panasci, *et al* (1987). They set up a study on the metabolic fate of tritriated Ac-[Nle^4,D-Phe^7]α-$MSH_{4-11}NH_4$ with the F1 variant of B16 melanoma cells. A 30cm Sephadex G-10 column was used to try and separate the various degraded products, and some success was achieved (Panasci, L.C. *et al*; 1987).

It is suffice to say that although the study reported here does prove the internalisation of [^{125}I-Tyr^2-Nle^4,D-Phe^7]α-MSH, and there is strong evidence for degradation occurring, much work involving degradation must be carried out to confirm, without any doubt, that this is the reason for the decrease in acid-resistant ^{125}I activity in the B16 melanoma cells after 30 minutes incubation at 37°C.

In parallel to the cell binding experiments just described, a series of subcellular fractionation experiments were designed. The aim of such were to follow the ligand once it had been internalised and hopefully monitor its fate; and in addition, if possible to quantify the amount of ligand intracellularly located. Even as early as 1971, DeDuve made detailed studies on the fractionation of tissues and cells. As far as the study here is concerned, the major compartments under scrutiny are the plasma membrane and the lysosome. The former, because, the plasma membrane is the site where the α-MSH receptor is located, and so ^{125}I activity associated with this area is extracellularly bound to the cells. The lysosome, is of great interest in respect of results obtained in the cell binding experiments in which degradation was strongly implied. Since the lysosome is otherwise referred to as the "degradative component of the cell", then it is assumed that this is the compartment where the ligand travels to along the endocytic pathway, prior to degradation.

In order to characterise the components under study, enzyme marker assays were introduced. The suitability of a particular enzyme for a cell type varies from cell to cell. During the course of the experiments, it was discovered that 5'-nucleotidase, a plasma membrane marker and β-glucuoronidase, a lysosomal marker were not sensitive enough to be measured for B16 melanoma cells. Instead alkaline phosphodiesterase I and β-hexosaminidase (plasma membrane and lysosomal markers respectively), were chosen.

Due to the fact that very little work has been documented up to date regarding the subcellular fractionation of B16 melanoma cells, a lot of "trial and error" was invovled. To begin with, the initial step in the whole process of subcellular fractionation is cell lysing, also known as homogenisation. In effect this is probably the most crucial step in the procedure mainly because the major aim for this study was to be able to isolate the plasma membrane and lysosomal components. An ideal homogenisation technique involves one

which separates the components so as not to disrupt internal component membranes. The two tools used were the needle and syringe method and the dounce homogeniser method- both depend on the force exerted by the operator. Although, for the method chosen, the needle and syringe method, it was standardised as far as possible in the fact that the syringe was pushed in 1 second each time. The cell lysis achieved was monitored by visualising the cells by use of the light microscope and incorporating a trypan blue dye which gives broken/disrupted cells a blue appearance. Each time, 90% lysis was used as the standard optimum for the cell lysis.

After homogenisation, the cells were subjected to a centrifugation step on a linear sucrose gradient in an attempt to separate the cellular components at different densities of sucrose. Fractionation of the density gradient was then carried out, and the fractions obtained analysed by enzyme marker assays to locate the position of the plasma membrane and lysosome components. In theory, if a method whereby efficient separation of these components can be achieved, then when a radioligand is added to the system, the resulting fractions can be monitored for ^{125}I activity and so the location of the iodinated ligand can be found.

Unfortunately this whole technique is not as simple as it seems. More often than not, satisfactory separation of the two components of interest was obtained. On the other hand, however, the plasma membrane component was not restricted to densities lower than for the lysosome. In effect, it was often observed in fractions normally associated with the lysosome. Due to this problem, it was obvious from early on that no quantitative measurements could be made. Another reason for this was that the entire procedure involves a large loss of radioactivity throughout the various stages of the experiment.

Even though a "perfect" system had not been developed as far as quantitative measurements were concerned, the system was adequate to determine the intracellular location of the [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH. Initial experiments which involved binding of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH to the cells were such that it could be clearly seen if internalisation at 37°C could be distinctly observed compared to no internalisation at 4°C. Indeed, this was the case and for the cells incubated at 37°C, a large amount of ^{125}I activity was associated with densities reflecting intracellular compartments. Acid-resistant ^{125}I activity contained a peak of activity between fractions 16 and 22 (densities 1.16 to 1.18gcm^{-3}); a similar peak was not found for the equivalent 4°C experiment.

Since it appeared possible to separate the internalised from surface-bound ^{125}I activity using cell fractionation, a series of timecourse experiments were designed. As for cell binding experiments, the ligand was allowed to bind to the cells for 2 hours at 4°C, at which point unbound radioligand was removed and the surface-bound ligand was permitted to enter the cells by incubation at 37°C. In general, the results obtained from these studies did show from the acid-washed data a gradual increase in ^{125}I activity around fractions 16 to 18 at 15 minutes. This was also apparent at the 30 minute timepoint, however, at 60 minutes ^{125}I activity present was not only very low, but it was spread widely over the gradient. At the first timepoint no internalised ligand is observed in the higher density lysosomal fractions due to surface binding occurring at 4°C. As the temperature was raised to 37°C, a gradual increase in ^{125}I activity could be seen in the higher density area corresponding to the lysosomal compartments. By 60 minutes, very little ^{125}I activity was associated with the cells at all. This could reflect degradation of the ligand and hence the disappearance of any radioactivity associated with the cells.

In view of the results obtained above, a parallel experiment was carried out, but, in the presence of 20mM NH_4Cl. Not surprisingly, this had the effect of maintaining a raised

level of ^{125}I activity within the cells as long as 60 minutes. Moreover, there was a peak of radioactivity present in the fractions relating to the lysosomal compartment. So it has been shown that the ligand binds to the receptor and moves inwards along the endocytic pathway to the lysosome where it remains if no degradation can occur due to inhibition of the degradative enzymes.

Again it must be emphasised that without an assay to identify degradative products for the [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH analogue, complete confirmation that the decrease in ^{125}I activity is associated with degradation. These cell fractionation studies do further imply such a phenomenon however and coincide with results obtained with cell binding experiments.

Therefore, as far as the sucrose gradient fractionation studies are concerned, at least one of the questions being challenged was answered somewhat. Unfortunately, until a more efficient and more consistent method of cell homogenisation is available quantitative analysis of the ^{125}I activity cannot be determined. Alternative gradient materials or centrifugation techniques are also a consideration to be kept in mind.

The final area of study was to consider the size of the ligand and observe its effect on the internalisation rate. In practice, the peptide will be the carrier, and a drug of higher molecular weight will be attached to the ligand. Therefore, even if the peptide [Nle^4,D-Phe^7]α-MSH itself can efficiently enter the cells it must be shown that when molecules of high molecular weight and larger size are attached to it, efficient internalisation still

occurs. What would be the advantage of having a carrier which transports a drug to the required site of action, but no mechanism of getting it inside the cell exists?

Therefore, the [Nle^4,D-Phe^7]α-MSH was in effect increased in size by the addition of biotin, and then streptavidin to the biotinylated peptide. For the former ligand, N$^\alpha$-biotin-[Nle^4,D-Phe^7]α-MSH, little difference was observed in the internalisation of the ligand. On the other hand however, the streptavidin- biotin-[Nle^4,D-Phe^7]α-MSH did not enter the cell as quickly. At the normal 90 minute period of the experiment, the ^{125}I activity was still shown to increasing. When the experiment was extended to 240 minutes, it was apparent that all the streptavidin-biotin-[Nle^4,D-Phe^7]α-MSH had entered the cells by 90 minutes and then a gradual decrease was observed. The major reason for the slowness of the streptavidin-biotin-[Nle^4,D-Phe^7]α-MSH uptake was probably due to the size of the streptavidin moiety. It is possible that it restricts the binding of the [Nle^4,D-Phe^7]α-MSH to the α-MSH receptor, hence, decreasing the efficiency of the entire system. Although only one set of data for this was available, it must be considered when adding drug molecules to the peptide that a decrease in rate of internalisation of the ligand-drug complex may occur.

Overall, the following points have been answered to some extent:-

1. It was confirmed that [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was bound to the α-MSH receptor on the cell surface and was subsequently internalised.
2. There was evidence to suggest from sucrose gradient fractionation studies that once within the cells, the ligand made its way to the lysosome.
3. Once the ligand reaches the lysosome, there is strong evidence from inclusion of ammonium chloride that degradation of the ligand occurs in the lysosome.

4. Inclusion of some protease inhibitors, leupeptin and pepstatin A, no inhibition of degradation was observed.
5. By adding larger moieties to the [^{125}I-Tyr^{2},Nle^{4},D-Phe^{e7}]α-MSH, the rate of internalisation was reduced.

In addition to the above areas where certain areas have been answered satisfactorily the following issues still need further confirmation:

1. Degradation does occur- it is necessary to develop an assay to isolate and recognise the degradative products
2. Further work on the subcellular fractionation - particularly the method of homogenising the B16 melanoma cells
3. Repeat of some of the protease enzyme inhibition enzyme work using a 10-fold higher concentration of the enzyme inhibitors
4. Further studies on addition of drugs to the Ac[Nle^{4},D-Phe^{7}]α-MSH ligand, and monitoring their internalisation rates
5. All this work has been carried out on B16 murine melanoma cells, parallel work on human melanoma cell lines need to be studied, followed by *in vivo* work

Although, it is apparent that there is still much scope of study left to do regarding the drug targeting of α-MSH analogues to melanoma cells, it is hoped that the work included in this thesis will be of some use to future workers in this area of research. In addition, it is necessary to bear in mind the similarity of MSH to other peptides, such as, ACTH and so care must be taken owing to the possibility of cross-recognition of the analogues. Furthermore, it is of the utmost necessity to establish if drug-analogue conjugates are capable of entering the cell and the drug can be released in a toxic form.

In conclusion, however, the specificity of peptides for their receptors indicate them to be extremely useful tools in selective drug delivery. Proof of their internalisation is also a step onwards, and sometime in the future a drug may be linked to a natural peptide and made use of clinically.

CHAPTER 7

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APPENDIX A

A1

Density, Refractive Index and Concentration Data for Sucrose at 20°C,
Molecular Weight = 342.3

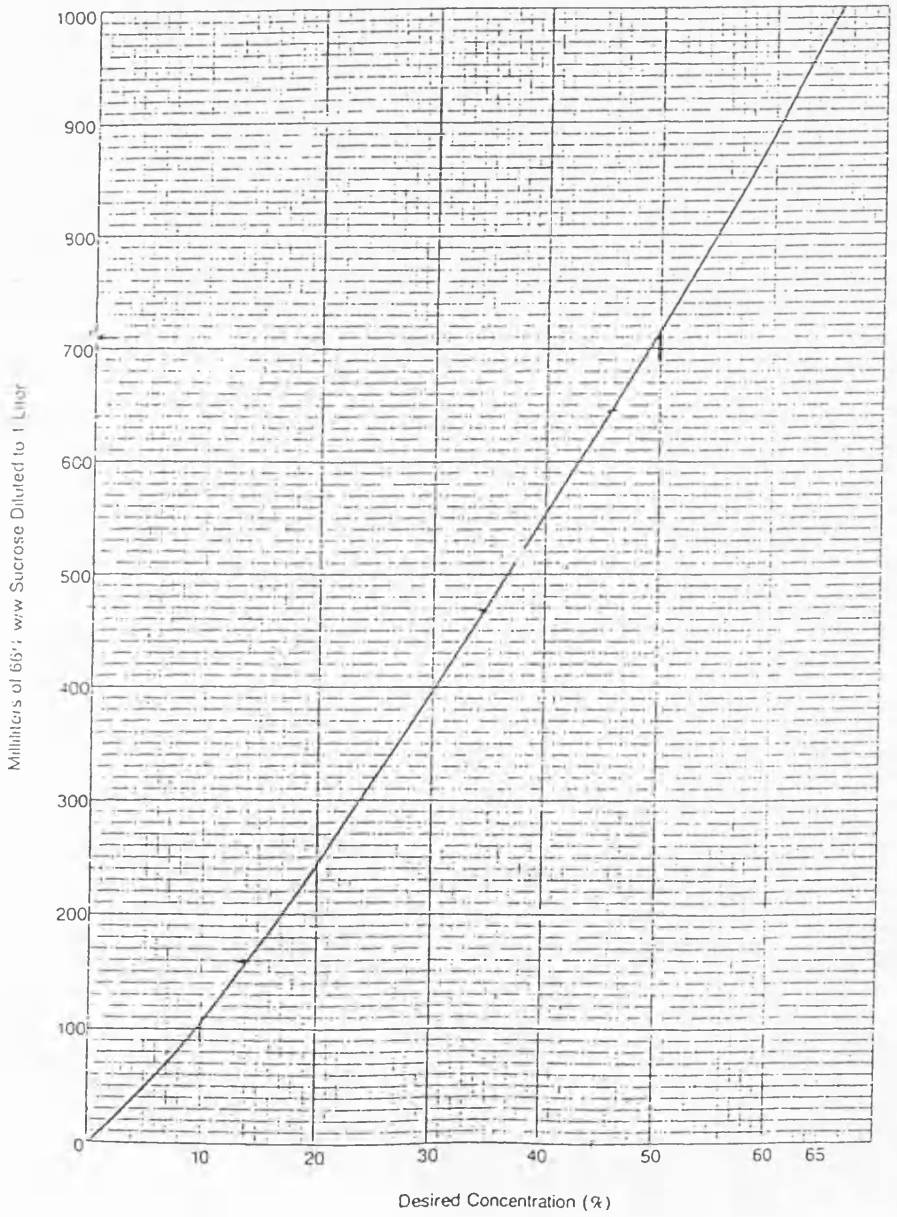
DENSITY (gcm^{-3})	REFRACTIVE INDEX, η_D	% BY WEIGHT	$mgml^{-1}$ OF SOLUTION	MOLARITY
0.9982	1.3330	0	-	-
1.0021	1.3344	1	10	0.029
1.0060	1.3359	2	20.1	0.059
1.0099	1.3374	3	30.3	0.089
1.0139	1.3388	4	40.6	0.119
1.0179	1.3403	5	50.9	0.149
1.0219	1.3418	6	61.3	0.179
1.0259	1.3433	7	71.8	0.210
1.0299	1.3448	8	82.4	0.211
1.0340	1.3464	9	93.1	0.272
1.0381	1.3479	10	103.8	0.303
1.0423	1.3494	11	114.7	0.335
1.0465	1.3510	12	125.6	0.367
1.0507	1.3526	13	136.6	0.399
1.0549	1.3541	14	147.7	0.431
1.0592	1.3557	15	158.9	0.464
1.0635	1.3573	16	170.2	0.497
1.0678	1.3590	17	181.5	0.530
1.0721	1.3606	18	193.0	0.564
1.0765	1.3622	19	204.5	0.597
1.0810	1.3639	20	216.2	0.632
1.0854	1.3655	21	227.9	0.666
1.0899	1.3672	22	239.8	0.701
1.0944	1.3689	23	251.7	0.735
1.0990	1.3706	24	263.8	0.771

DENSITY (gcm^{-3})	REFRACTIVE INDEX, η_D	% BY WEIGHT	$mgml^{-1}$ OF SOLUTION	MOLARITY
1.1036	1.3723	25	275.9	0.806
1.1082	1.3740	26	288.1	0.842
1.1128	1.3758	27	300.5	0.878
1.1175	1.3775	28	312.9	0.914
1.1222	1.3793	29	325.4	0.951
1.1270	1.3811	30	338.1	0.988
1.1318	1.3829	31	350.9	1.025
1.1366	1.3847	32	363.7	1.063
1.1415	1.3865	33	376.7	1.100
1.1463	1.3883	34	389.7	1.138
1.1513	1.3902	35	403.0	1.177
1.1562	1.3920	36	416.2	1.216
1.1612	1.3939	37	429.6	1.255
1.1663	1.3958	38	443.2	1.295
1.1713	1.3978	39	456.8	1.334
1.1764	1.3997	40	470.6	1.375
1.1816	1.4016	41	484.5	1.415
1.1868	1.4036	42	498.5	1.456
1.1920	1.4056	43	512.6	1.498
1.1972	1.4076	44	526.8	1.539
1.2025	1.4096	45	541.1	1.581
1.2079	1.4117	46	555.6	1.623
1.2132	1.4137	47	570.2	1.666
1.2186	1.4158	48	584.9	1.709
1.2241	1.4179	49	599.8	1.752
1.2296	1.4200	50	614.8	1.796
1.2351	1.4221	51	629.9	1.840
1.2406	1.4242	52	645.1	1.885
1.2462	1.4264	53	660.5	1.930
1.2519	1.4285	54	676.0	1.975

DENSITY (gcm^{-3})	REFRACTIVE INDEX, η_D	% BY WEIGHT	$mgml^{-1}$ OF SOLUTION	MOLARITY
1.2575	1.4307	55	691.6	2.020
1.2632	1.4329	56	707.4	2.067
1.2690	1.4351	57	723.3	2.113
1.2748	1.4373	58	739.4	2.160
1.2806	1.4396	59	755.6	2.207
1.2865	1.4418	60	771.9	2.255
1.2924	1.4441	61	788.3	2.303
1.2983	1.4464	62	788.3	2.351
1.3043	1.4486	63	804.9	2.401
1.3103	1.4509	64	821.7	2.450
1.3163	1.4532	65	855.6	2.500
1.3224	1.4558	66	872.8	2.550
1.3286	1.4581	67	890.2	2.864

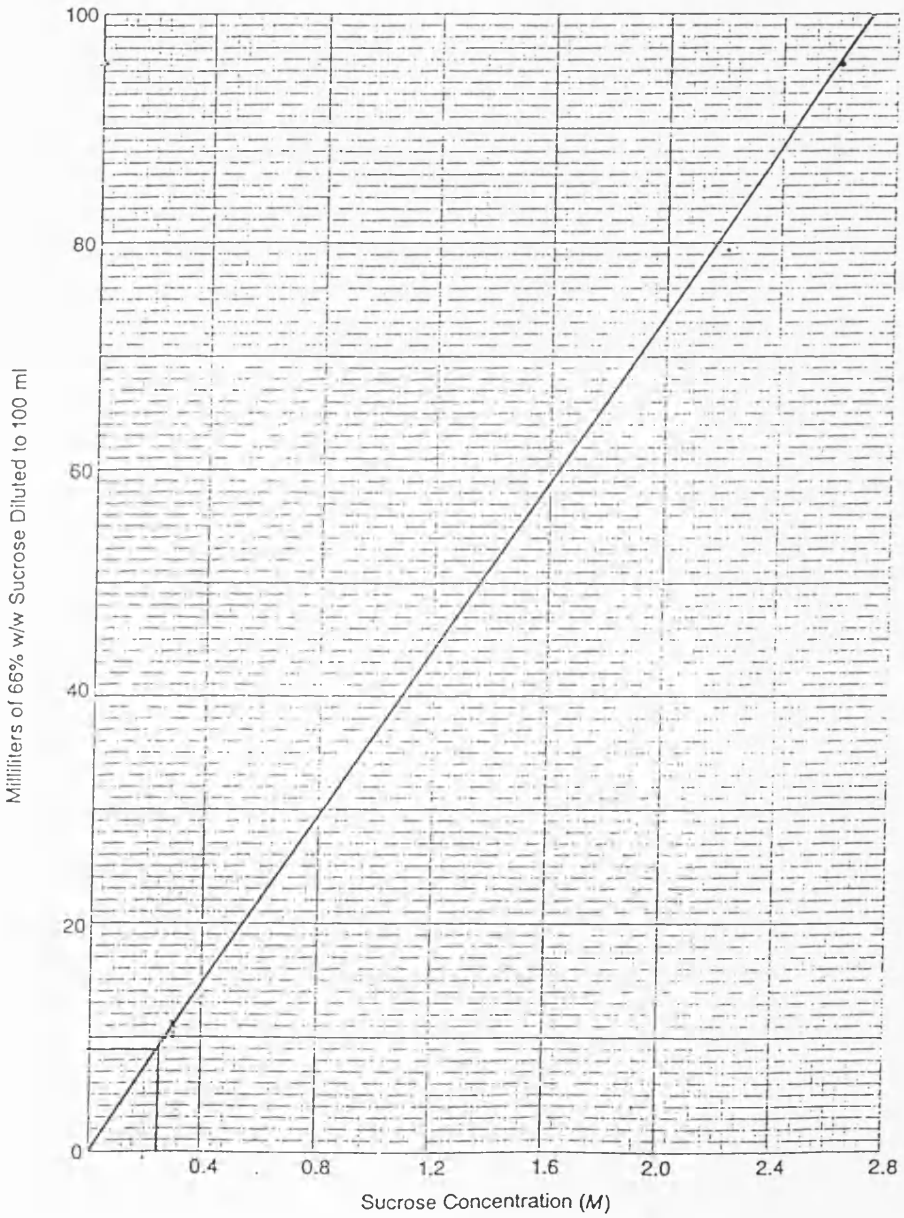
A2

Sucrose Dilution Chart For %(w/w) Concentrations



A3

Sucrose Dilution Chart For Molar Concentrations



APPENDIX B

EXPERIMENTAL DATA FOR CHAPTER 3

B1 Binding and Internalisation of 0.1nM [^{125} I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH with B16 Melanoma Cells at 4°C with Time in a HEPES Based Buffer, pH 7.4 (n=4)

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
15	2000	107	326	7	1674	107	165	43
30	2804	285	418	27	2386	286	214	35
60	4474	306	420	53	4054	310	228	33
90	5606	529	507	48	5099	531	410	112
120	6468	621	666	86	5802	626	437	67
240	10614	631	1077	90	9537	637	713	100

B2 Binding and Internalisaton of 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-PHE^{7}]α-MSH with B16 Melanoma Cells at 4°C with Time in a HEPES Based Buffer, pH 7.4 (n=4)

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
15	670	66	521	68	149	94	219	44
30	823	97	473	70	350	119	210	14
60	1211	149	737	137	474	202	221	74
90	1682	130	1002	139	680	190	232	19
120	1753	136	952	166	801	214	269	30
240	2278	294	1243	104	1035	311	404	29

B3 Binding and Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with B16 Melanoma Cells at 37°C in the Absence of 20mM NH_4Cl with Time in a HEPES Based Buffer, pH 7.4 (n=4)

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
15	3760	370	312	58	3448	374	435	23
30	8940	505	366	70	8574	509	2857	265
60	13202	1077	522	69	12680	1079	5201	62
90	12946	452	461	36	12485	453	5267	293
120	11091	565	511	42	10581	566	4681	638
240	6078	196	836	52	5242	202	3790	85

B4 Binding and Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with B16 Melanoma Cells at 37°C in the Absence of 20mM NH_4Cl with Time in a HEPES Based Buffer, pH 7.4 (n=4)

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
15	2838	346	261	42	2577	348	310	24
30	5580	608	369	22	5211	608	1101	111
60	7720	345	330	15	7390	345	2894	183
90	7050	322	396	66	6654	328	2666	174
120	6538	240	374	39	6164	243	2552	131
240	4426	546	369	30	4057	546	2414	87

B5 Binding and Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with B16 Melanoma Cells at 37°C in the Presence of 20mM NH_4Cl with Time in a HEPES Based Buffer, pH 7.4 (n=4)

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
15	5179	236	383	31	4796	238	872	50
30	9491	388	283	30	9208	389	3553	68
60	14109	80	397	270	13712	84	7965	313
90	19726	674	413	22	19313	674	13300	1144
120	21862	2074	544	61	21318	2074	15973	1285
240	28917	1568	866	39	28051	1568	25868	700

B6 Binding and Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with B16 Melanoma Cells at 37°C in the Presence of 20mM NH_4Cl with Time in a HEPES Based Buffer, pH 7.4 (n=4)

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
15	5966	438	306	28	5660	438	793	83
30	12132	1056	267	19	11865	1056	4073	491
60	20740	708	407	21	20333	708	11282	470
90	28346	1834	402	32	27944	1834	18840	1343
120	33135	3423	563	26	32572	3423	23308	1391
240	47362	3467	959	32	46403	3467	38511	1077

B7 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	4408	73	312	39	4096	83	25	39
5	3436	358	95	53	3341	361	560	57
10	2785	323	73	20	2712	323	791	114
20	3014	270	58	12	2956	270	1055	40
30	2975	76	59	9	2916	76	1225	21
45	2465	147	52	25	2413	149	910	70
90	1569	230	38	21	1531	231	391	66

B8 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	6662	391	477	70	6185	397	718	258
5	6184	163	142	35	6042	166	920	238
10	6039	384	96	18	5943	384	1228	166
20	5186	348	156	94	5030	360	1431	158
30	5197	402	89	15	5108	402	2387	170
45	4329	339	109	53	4220	343	2186	145
90	2101	555	87	37	2014	556	973	301

B9 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4)in the Presence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	2513	113	328	7	2185	113	283	42
5	1994	248	157	11	1837	248	242	21
10	1614	209	141	31	1473	211	357	41
20	1545	139	114	34	1431	143	469	36
30	1974	144	121	27	1853	146	693	108
45	1602	108	105	21	1497	110	875	84
90	1688	246	76	4	1612	246	1585	151

B10 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	2395	179	706	5	1689	179	399	71
5	1550	107	271	8	1279	107	298	105
10	1333	155	221	19	1112	156	302	26
20	1272	157	178	15	1094	158	328	16
30	1131	46	111	10	1020	47	471	18
45	982	85	83	15	899	86	587	5
90	950	57	64	15	886	59	932	24

B11 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of Leupeptin

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	3220	162	91	61	3129	173	109	10
5	2603	185	89	18	2514	185	253	26
10	2443	230	44	12	2399	230	430	39
20	2692	108	63	3	2629	108	854	46
30	2080	169	32	23	2048	170	1213	53
45	2008	60	37	8	1971	60	1019	58
90	879	230	21	13	858	230	435	64

B12 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of Leupeptin

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	1389	61	347	65	1263	72	121	10
5	1263	72	109	7	1154	72	153	7
10	1460	201	46	26	1414	202	247	39
20	1250	65	63	15	1187	66	438	36
30	1140	89	35	25	1105	92	538	68
45	903	69	42	2	861	69	386	23
90	400	34	21	14	379	36	230	27

B13 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of Leupeptin

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	3921	658	144	17	3777	658	148	13
5	3254	285	102	20	3152	285	437	36
10	3284	257	113	38	3171	257	592	38
20	3042	249	81	18	2961	249	1118	69
30	3119	18	31	7	3088	19	1432	83
45	2488	141	68	18	2420	142	1203	152
90	1147	140	27	14	1120	140	670	22

B14 Internalisation of 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of Leupeptin

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	2310	64	658	61	1652	88	233	21
5	1272	135	123	9	1149	135	193	21
10	1644	275	104	19	1540	275	336	29
20	1534	75	102	25	1432	79	748	61
30	1272	24	71	25	1201	34	637	49
45	810	191	56	22	754	192	410	24
90	481	103	51	24	430	105	291	54

B15 Internalisation of 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of Pepstatin A

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	3440	219	310	45	3130	223	128	11
5	2795	251	86	8	2709	251	254	16
10	2909	359	73	11	2836	359	379	65
20	2513	148	63	16	2450	148	618	70
30	1969	68	10	5	1959	68	941	43
45	1947	344	54	23	1893	344	890	75
90	1259	383	36	7	1223	383	501	46

B16 Internalisation of 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of Pepstatin A

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	2974	128	677	151	2297	197	242	20
5	1562	95	160	73	1402	119	238	29
10	1386	184	172	13	1214	184	326	161
20	986	67	61	13	925	68	495	61
30	1066	84	26	11	1040	84	529	29
45	655	86	60	8	595	86	409	19
90	336	58	17	5	319	58	210	30

B17 Internalisation of 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of Pepstatin A

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	3123	369	324	18	2799	369	151	21
5	2795	230	84	19	2711	230	379	92
10	2675	211	88	8	2587	211	550	27
20	2375	255	57	9	2318	255	713	20
30	1921	210	21	16	1900	210	933	37
45	1672	235	62	34	1610	237	809	115
90	1056	364	42	39	1014	366	548	135

B18 Internalisation of 0.1nM [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of Pepstatin A

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	2427	121	661	49	1766	130	263	37
5	1611	77	114	17	1497	78	199	27
10	1561	456	143	46	1418	162	312	44
20	1529	78	130	19	1399	80	676	43
30	1197	44	42	24	1155	50	606	31
45	1047	31	108	24	939	39	586	100
90	404	82	39	18	365	83	281	46

APPENDIX C

EXPERIMENTAL DATA FOR CHAPTER 4

C1 Density of fractionated linear sucrose gradients against fraction number

FRACTION NUMBER	REFRACTIVE INDEX	DENSITY (gcm^{-3})
1	1.349	1.042
2	1.356	1.059
3	1.357	1.064
4	1.360	1.072
5	1.364	1.081
6	1.366	1.085
7	1.369	1.094
8	1.369	1.094
9	1.372	1.104
10	1.374	1.108
11	1.376	1.113
12	1.377	1.118
13	1.381	1.127
14	1.383	1.132
15	1.385	1.137
16	1.387	1.142
17	1.390	1.151
18	1.392	1.156
19	1.394	1.161
20	1.398	1.171
21	1.400	1.176
22	1.403	1.187
23	1.406	1.192
24	1.408	1.197
25	1.410	1.203

C2 β-Hexosaminidase activity present in the sucrose fractions of the densities shown (Note, the enzyme assay was performed on alternate sucrose fractions due to the amount of sample there was available)

FRACTION NUMBER	DENSITY (gcm^{-3})	ABSORBANCE (λ_{400nm})
2	1.055	0.347
4	1.067	0.248
6	1.081	0.098
8	1.094	0.057
10	1.108	0.064
12	1.118	0.099
14	1.132	0.182
16	1.142	0.640
18	1.156	1.366
20	1.171	0.135
22	1.187	0.801
24	1.197	0.135

C3 Alkaline Phosphodiesterase I activity present in the sucrose fractions of the densities shown
(Note, the enzyme assay was performed on alternate sucrose fractions due to the amount of sample there was available)

FRACTION NUMBER	DENSITY (gcm^{-3})	ABSORBANCE (λ_{410nm})
2	1.042	0.436
4	1.063	0.784
6	1.081	0.285
8	1.091	0.271
10	1.103	0.228
12	1.113	0.221
14	1.127	0.213
16	1.136	0.197
18	1.151	0.234
20	1.161	0.346
22	1.176	0.285
24	1.192	0.250

C4 ^{125}I Activity associated with each sucrose fraction for cell incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH prior to homogenisation and centrifugation.

FRACTION NUMBER	TOTAL BINDING (CPM)	NON-SPECIFIC BINDING (CPM)	ACID WASHED CELLS (CPM)
1	482	216	127
2	470	204	126
3	403	179	90
4	284	82	60
5	191	86	33
6	162	52	29
7	138	28	13
8	125	26	18
9	99	11	9
10	149	4	4
11	141	11	13
12	136	11	14
13	137	10	9
14	130	10	10
15	144	8	24
16	138	6	23
17	160	15	18
18	163	15	21
19	185	11	24
20	247	19	15
21	266	28	15
22	262	17	11
23	232	15	8
24	220	15	7
25	226	13	6

C5 ^{125}I Activity associated with each sucrose fraction for cell incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH followed by 30 minutes incubation at 37°C prior to homogenisation and centrifugation.

FRACTION NUMBER	TOTAL BINDING (CPM)	NON-SPECIFIC BINDING (CPM)	ACID-WASHED CELLS (CPM)
1	729	143	327
2	677	135	283
3	552	97	215
4	463	59	174
5	437	42	110
6	511	24	81
7	690	14	73
8	785	19	75
9	829	12	103
10	843	6	119
11	764	10	129
12	762	9	142
13	723	6	170
14	733	13	200
15	804	15	320
16	943	15	431
17	1317	17	636
18	1870	22	672
19	2090	26	548
20	1116	27	697
21	1246	35	331
22	1169	23	277
23	1012	17	98
24	771	10	102
25	308	15	79

C6 ^{125}I activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH followed by the relevant times incubation at 37°C prior to homogenisation and fractionation.

FRACTION NUMBER	ONLY AT 4°C		AFTER 15 MINUTES AT 37°C	
	TOTAL BINDING (CPM)	ACID WASHED (CPM)	TOTAL BINDING (CPM)	ACID WASHED (CPM)
1	483	6	147	45
2	471	15	131	46
3	419	2	140	37
4	363	32	153	19
5	278	20	112	14
6	201	8	93	6
7	142	5	112	7
8	136	0	117	8
9	124	0	139	14
10	148	8	128	27
11	127	6	154	30
12	128	9	142	20
13	141	20	138	27
14	147	7	144	15
15	131	11	167	26
16	169	0	195	30
17	186	7	222	45
18	242	15	241	69
19	325	20	240	50
20	358	26	165	41
21	278	20	124	30
22	196	1	90	9
23	204	17	59	1
24	181	4	39	13
25	144	0	54	20

C6 (continued) ^{125}I activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH followed by the relevant times incubation at 37°C prior to homogenisation and fractionation.

FRACTION NUMBER	AFTER 30 MINUTES AT 37°C		AFTER 60 MINUTES AT 37°C	
	TOTAL BINDING (CPM)	ACID WASHED (CPM)	TOTAL BINDING (CPM)	ACID WASHED (CPM)
1	81	36	39	15
2	90	16	33	0
3	63	13	15	8
4	72	2	13	12
5	57	1	16	17
6	55	9	28	23
7	81	28	40	19
8	58	21	32	15
9	80	40	25	13
10	90	30	19	0
11	82	35	6	0
12	84	32	18	0
13	76	36	35	6
14	85	54	22	21
15	111	52	43	20
16	94	80	35	8
17	150	75	26	11
18	303	46	25	6
19	103	42	18	0
20	75	15	17	16
21	41	10	18	7
22	30	15	20	18
23	44	13	23	19
24	50	39	24	8
25	27	19	8	2

C7 ^{125}I activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH followed by the relevant times incubation at 37°C in the presence of 20mM NH_4Cl, prior to homogenisation and fractionation.

FRACTION NUMBER	ONLY AT 4°C		AFTER 15 MINUTES AT 37°C	
	TOTAL BINDING (CPM)	ACID WASHED (CPM)	TOTAL BINDING (CPM)	ACID WASHED (CPM)
1	491	37	439	37
2	447	43	384	43
3	403	44	364	44
4	643	39	292	39
5	466	35	228	35
6	328	33	200	33
7	274	12	214	14
8	229	30	166	12
9	220	32	190	43
10	183	45	203	43
11	186	44	196	45
12	196	44	182	52
13	212	47	176	44
14	198	44	203	47
15	164	49	224	44
16	242	35	182	52
17	250	34	176	44
18	437	44	203	47
19	506	50	224	44
20	460	27	272	49
21	398	17	355	73
22	377	12	412	69
23	406	7	443	88
24	525	18	559	198
25	343	14	283	55

C7 (continued) ^{125}I activity associated with each sucrose fraction for cells incubated for 2 hours at 4°C with 0.1nM [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH followed by the relevant times incubation at 37°C in the presence of 20mM NH_4Cl, prior to homogenisation and fractionation.

FRACTION NUMBER	AFTER 30 MINUTES AT 37°C		AFTER 60 MINUTES AT 37°C	
	TOTAL BINING (CPM)	ACID WASHED (CPM)	TOTAL BINDING (CPM)	ACID WASHED (CPM)
1	287	216	97	337
2	254	184	65	191
3	227	119	52	115
4	186	88	27	91
5	172	35	43	77
6	182	27	42	68
7	183	57	52	48
8	213	55	51	29
9	180	60	45	24
10	194	75	53	23
11	162	44	40	27
12	157	79	53	25
13	148	71	60	42
14	193	140	96	68
15	260	158	96	82
16	321	71	160	106
17	477	140	269	154
18	477	158	452	156
19	497	228	653	258
20	381	264	549	348
21	293	284	419	376
22	227	275	328	311
23	186	247	266	189
24	234	203	284	156
25	277	171	194	125

APPENDIX D

EXPERIMENTAL DATA FOR CHAPTER 5

D1 Internalisation of Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	4692	313	1128	43	3563	315	190	36
5	3248	352	178	28	3070	353	594	57
10	2882	469	129	26	2752	469	675	76
20	2229	235	107	29	2122	236	668	32
30	1272	212	101	39	1171	215	549	102
45	1330	164	58	19	1272	165	470	65
90	993	198	53	22	940	199	447	41

D2 Internalisation of Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16Melanoma Cells with time (n=4) in the Absence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	1789	282	1356	55	433	287	15	30
5	1241	76	862	56	379	94	341	64
10	1121	111	766	93	355	144	404	987
20	867	77	558	14	309	78	387	10
30	534	73	209	28	325	78	30	54
45	479	80	140	17	335	81	64	37
90	384	29	84	3	300	29	347	17

D3 Internalisation of Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	3672	113	448	17	3225	114	30	18
5	2577	211	123	16	2454	211	233	20
10	2321	213	88	16	2234	213	368	27
20	2467	183	111	11	2356	183	552	50
30	2261	58	185	100	2076	115	596	35
45	2252	98	64	15	2192	99	786	97
90	2163	87	40	5	2132	87	1422	46

D4 Internalisation of Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	5585	371	1120	106	4465	385	200	50
5	4194	129	250	42	3944	135	556	47
10	4044	216	190	19	3854	216	676	7
20	3674	138	158	14	3516	138	850	47
30	3483	79	126	17	3357	80	1062	92
45	339	178	111	7	3228	178	1503	47
90	3268	206	99	28	3169	207	2296	110

D5 Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	1786	204	374	14	1412	204	80	18
5	1301	319	139	29	1162	320	289	37
10	890	112	127	14	763	113	347	51
20	802	76	106	9	696	76	481	26
30	931	12	92	20	839	23	505	24
45	736	115	159	42	577	122	509	12
90	349	80	72	12	277	81	695	12

D6 Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Absence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	2267	127	556	21	1711	128	77	17
5	1589	100	121	15	1468	101	266	25
10	1345	69	88	23	1257	73	301	7
20	1316	45	57	13	1259	46	512	48
30	1117	158	75	16	1042	158	536	37
45	1036	71	68	14	968	72	645	60
90	438	50	19	19	419	53	739	154

D7 Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time in the Absence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	1777	161	833	74	944	177	89	38
5	833	42	118	16	715	44	201	36
10	726	99	94	15	632	100	267	35
20	537	93	55	11	482	93	194	97
30	414	63	31	15	383	64	276	41
45	514	142	23	13	491	142	444	32
90	335	46	3	3	332	46	601	60
240	169	30	14	10	155	31	434	11

D8 Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time in the Presence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	2256	187	573	22	1683	188	44	10
5	1619	54	150	7	1469	54	415	45
10	1406	33	96	8	1310	33	481	26
20	1347	208	80	11	1267	208	582	36
30	1157	131	94	20	1063	132	619	59
45	1000	23	47	24	953	33	658	49
90	1047	94	24	5	1023	94	830	80

D9 Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	1822	33	411	34	1411	47	58	35
5	1358	72	225	33	1134	79	375	44
10	1147	146	187	20	960	147	313	40
20	1068	72	146	17	922	73	497	61
30	1127	68	133	13	994	69	582	33
45	933	90	133	17	800	91	618	30
90	936	37	102	15	834	39	871	95

D10 Internalisation of Streptavidin-Biotin-[^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH at 37°C after binding for 2 hours at 4°C into B16 Melanoma Cells with time (n=4) in the Presence of 20mM NH_4Cl

TIME (MINS)	TOTAL BINDING		NON-SPECIFIC BINDING		SPECIFIC BINDING		INTERNALISED LIGAND	
	CPM	±	CPM	±	CPM	±	CPM	±
0	1822	251	586	13	1236	251	328	39
5	1273	131	186	30	1087	134	463	47
10	981	43	111	11	870	44	511	30
20	827	67	77	11	750	67	563	113
30	751	162	45	29	706	164	585	44
45	824	125	54	8	770	125	605	61
90	951	31	26	19	925	36	849	80
240	844	109	72	28	772	112	914	90